Cholinergic mechanisms and behaviour in selected strains of rats.

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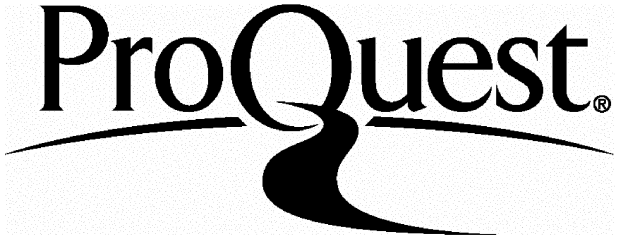

'All animals are equal, but some animals are more equal than others.'

George Orwell (Animal Farm)

SUMMARY

The main aim of this work was to investigate the role of central cholinergic mechanisms in the control of behaviour. Three strains of rat, which display behavioural differences, were chosen for use in the work, these were the Roman High Avoidance (RHA) and the Roman Low Avoidance (RLA) strains, with the Porton strain to act as a reference line. Differences in conditioned avoidance behaviour and spontaneous activity were demonstrated between the strains and also in their behavioural response to drugs known to affect the central cholinergic system. These were, an anti-acetylcholine drug, N-ethyl-3-piperidyl benzilate which produced hyperactivity and facilitated avoidance conditioning, an anti-cholinesterase drug, physostigmine, which depressed spontaneous activity and conditioned avoidance behaviour. No change in behaviour of any of the strains was seen after treatment with drugs thought to have mainly peripheral actions on the cholinergic system. These drugs were N-ethyl-3-piperidyl benzilate methiodide and pyridostigmine. Strain differences were also seen in response to an adrenergic drug, d-Amphetamine.

Facilitated behavioural performance was seen after a combination of anti-acetylcholine and adrenergic drugs were given to rats, which when given separately, at the same doses, produced no effect on behaviour.

Determination of acetylcholine concentration and cholinesterase activity in discrete brain areas revealed differences in the former but not the latter activity, implying that differences also exist between the strains in enzyme to substrate ratios.

The support these findings give to theories of cholinergic inhibition in the control of behaviour and to the idea that behavioural control results from the central interaction of cholinergic and adrenergic transmitter systems is discussed.

The value of selectively bred strains as tools in psychopharmacology is also assessed in the light of this work.

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CONTENTS

CHAPTER 1

Introduction

1.1 Inheritance of Behaviour and the Use of Strains

Many aspects of behaviour are subject to genetic control and are inherited therefore, according to the same laws of genetics as other characters of the phenotype. This fact has been exploited by animal breeders over the centuries who, whether desiring, for instance, docility in farm animals, or hunting ability in dogs, have selectively bred for these characters in their stock, as well as for high milk yields or for coat colour. Similarly human behavioural traits tend to run in families suggesting an inherited component of human behaviour. The nature of inherited behaviour has recently received much attention and as a result more is now known of its mechanisms. It has become apparent, for example, that behaviour patterns are inherited via complex polygene systems and in general, behaviour shows continuous or quantitative variation, rather than discontinuous variation. Discontinuous variation is the type exhibited by phenotypes such as eye colour or body size, where a discrete manifestation is apparent, controlled by a relatively small gene number and subject to simple Mendelian analysis. Continuous variation is more difficult to analyse but the development of biometrical genetics (Mather 1949) has permitted the mathematical analysis of some aspects of the inheritance of behaviour, (Broadhurst 1960; Broadhurst and Jinks 1961).

A complicating factor in studies of behaviour inheritance is the part played by the environment in modifying the behavioural characteristics of the individual. It is axiomatic that the expression of a

given genotype will vary according to the environment in which it is allowed to develop and the behaviour exhibited is always the result of this complex interaction. The part played by each factor may vary, thus an inborn error of metabolism may be manifested under all environmental conditions whilst another subtle genetic component may only be apparent when certain environmental conditions are present. Much controversy still exists as to the relative importance of these two controlling factors especially when human behaviour and intelligence are concerned. Because of a lack of controlled data, discussions of this problem in humans is often only speculative. Even in the animal laboratory where controlled environments and breeding experiments are possible the investigation of this complex interaction is difficult.

Investigations of the inheritance of animal behaviour in the laboratory requires carefully maintained and fully controlled environmental conditions so that variation from this source is reduced to a minimum. Each generation must experience identical conditions of ambient temperature, light-dark cycle, diet, noise level and general husbandry, including such factors as the number of animals kept in a cage, the amount of handling received and the times of feeding and cage cleaning. By altering these parameters, one at a time, it is possible to examine their relative importance in behaviour. Broadhurst (1958) examined the effects of handling, previous experimental experience and sexual experience on emotionality in rats when measured in the open-field. Unlike Weininger (1954), he found no effects due to handling on this behaviour but found significant effects attributable to the 'experience' parameters. The inherent variability of behaviour seen in animal populations, even those kept in controlled environments, has been a source of interest to those investigating the genetic

control of behaviour and a source of frustration to those attempting to use animals as tools to investigate the toxicological actions of drugs. Behavioural performance may vary considerably between individuals within a randomly bred strain tested for a particular character. Bovet, Bovet-Nitti and Oliverio (1969) measured rates of learning of a conditioned avoidance response by a Swiss strain of mice over several training sessions and observed that a distinctly different level of performance was attained by each individual. This is a common finding in non-inbred populations and has been noted by many workers. Bignami (1962) Bovet, Bignami and Robustelli (1963) and Bignami (1964) all refer to the use of litter-mate controls in behavioural tests, in an effort to reduce the wide variation present, complicating drug studies. Comparisons between randomly bred strains can reveal large variations in performance, thus Nakamura and Anderson (1962), examined shock avoidance learning in Sprague Dawley albino strain and Long-Evans hooded rats. Subjects were trained to turn a wheel as the avoidance response and in this test, the hooded rats were superior to the albinos. Variation within inbred strains, as one may expect, is much smaller than in randomly bred strains of mice and rats, but differences in performance between inbred strains may be large. Bovet et al (1969) compared inbred strains of mice trained in conditioned avoidance and found that although there was good consistency within a strain, even strains which were closely related, having been produced by crossings with a common strain, differed considerably in performance. Stamm (1954) reported differences in hoarding behaviour between homozygous strains of rat and also differences in emotionality levels. Broadhurst (1958) looking at genetic components in the control of emotionality in these and other strains of wild and inbred laboratory rats, found variations in emotionality

between strains, which apparently correlated with the coat colour of the animals. Other workers have also noted behavioural correlations with coat colour, Keeler (1948) and Winston, Lindzey and Connor (1967). Winston et al found albino rats to be poor active avoidance performers when compared with pigmented rats but found no such difference when compared for passive-avoidance learning. They suggested that albinism may be gene-linked with the behavioural tendency to 'freeze' in aversive situations, leading to poor active-avoidance. This is an interesting observation, since many laboratories choose albino rats as material for behavioural experiments involving aversive stimuli. As behavioural differences between strains are often so great, it would seem to be a basic requirement that, before embarking on programmes of pharmacological work, the strain as well as the species, be carefully chosen according to performance in a particular type of test. Inbred strains or litter-mates would seem to be useful in this context, where consistency of performance is imperative and there is also, clearly, evidence to suggest that pigmented or hooded rats may be superior in learning tests to albinos.

Strains

Where the study of the genetics of behaviour has been perhaps, most fruitful is in the field of selectively bred strains.

1.11 Tryon Strains

The first extensive work done in this field was by Tryon (1940). Tryon selectively bred, on the basis of error scores in a complex alley-maze, two strains of rats which differed in their ability to run the maze. After several generations of selection two distinct strains were produced which became known as the Tryon S1, maze-brights, which learned

the maze quickly with few error scores, and the Tryon S3, maze-dulls, which learned the maze slowly and with many errors. Thus Tryon produced two new strains which possessed in exaggerated form, the extremes of maze-learning ability that had existed in the original populations. Furthermore, these two strains could be bred as pure lines without further selection occurring. These strains have provided valuable material for the study of behavioural genetics, and descendants of these strains have been used by several workers, Searle (1949), Rosenzweig, Krech and Bennett (1960), Rowland and Woods (1961), McGaugh, Jennings and Thomson (1962) and Tapp (1964). Subsequent work with the Tryon strains has shown that the selection procedure, based purely on the error scores in the maze, produced strains that did not necessarily differ, either in general learning ability, or even in the ability to run alley-mazes but more particularly, in the ability to run the type of maze that was employed in the selection experiments (Searle 1949). Searle was able to demonstrate that the differences in error scores attained by the 'maze-brights' and 'maze-dulls' when they were trained in a swim-maze were negligible and there was even a tendancy for the 'maze-dulls' to be superior. Searle suggested that rather than a difference in learning ability they demonstrated differences in motivation and emotionality levels. The original maze used food reward and Searle was able to show in other tests, that the 'maze-brights' had a higher food motivational level than the 'maze-dulls'. When tested in an open-field situation, the 'maze-brights' demonstrated a higher level of emotionality than their counterparts, and when tested for spontaneous locomotor activity showed lower activity levels than the 'maze-dulls'. Thus the 'maze-brights' with high emotionality and reduced exploratory activity, show an 'economy of movement' which, therefore, may lead to fewer errors which was the original criterion for selection. Tapp (1964) demonstrated higher emotionality levels in the maze-brights than

the maze-dulls when he compared the performance of the strains in a conditioned emotional response. This confirms part of Searle's work but it may be worth noting in this context that the modern descendants of these strains may no longer be breeding true for their original characters. Rowland and Woods (1961) report several differences between the Tryon strain descendants and the original strains.

1.12 Strains selected for brain biochemistry

One line of research that has stemmed in part from the Tryon strain experiments, has been the work of Rosenzweig, Krech and Bennett (1960) who used descendants of the strains in their search for correlations between brain biochemistry and behaviour. They discovered a further difference between the 'maze-brights' and 'maze-dulls', this time a chemical one; there was a significant difference in the activity levels of acetylcholinesterase in the brain and in the quantities of its substrate, acetylcholine between the two strains. Previous work by the team Krech, Rosenzweig, Bennett and Kruekel (1954), had demonstrated strain differences in cerebro-cortical cholinesterase activity of the rat which was apparently correlated with behavioural characteristics. Like the Tryon strains these animals had demonstrated differences in maze learning ability but in this case the measures were of 'adaptive behaviour' in a maze where the correct solution was occasionally changed. Animals which showed good'adaptive behaviour' were found to have higher cortical cholinesterase activity than those showing poor 'adaptive behaviour' and were compared, therefore, with the Tryon maze-brights. To investigate further the role of cholinesterase and acetylcholine in behaviour control, the same laboratory embarked on a series of selective breeding experiments (Roderick 1960). Roderick bred from two stock populations of rats, four new strains which differed in levels of cholinesterase activity in the cortex. From each population he produced two strains selected for either high or low cholinesterase activity

in the visual or somesthetic cortex. These cortical areas were chosen because Rosenzweig et al (1960) had been interested in rats which had 'visually' or 'spatially' orientated preferences in learning the 'adaptive behaviour' maze-test. The strains produced were known as the Roderick Dempster High (RDH) and Roderick Dempster Low (RDL), Roderick Castle High (RCH) and Roderick Castle Low (RCL), where 'high' and 'low' refer to levels of cholinesterase activity. There were significant correlations between behaviour in adaptive maze situations and cholinesterase activities in these strains but not in the direction that was predicted from the work with Tryon strains. Later when these and the Tryon strains were examined for levels of acetylcholine in the brain, (Bennett, Crossland, Krech and Rosenzweig, 1960), it was discovered that the ratio of enzyme to substrate had been changed in the Roderick strains, and in fact, the levels of acetylcholine had remained similar to the parent stocks. Selection for the Tryon strains was based on behavioural measures and since levels of cholinesterase and acetylcholine had been similarly altered the cholinesterase : acetylcholine ratio in the brain, had been maintained. Proof that the cholinesterase and acetylcholine content of brain are not necessarily inherited via a common gene system was thus obtained. Later, further selective breeding experiments in the same laboratory (Bennett, Herbert, Rosenzweig and Krech, 1964) produced strains that were selected for levels of acetylcholine respectively. This time the ratio was maintained, cholinesterase activities being raised or lowered in parallel with acetylcholine and when tested behaviourally it was found that these animals resembled, at least in maze-learning ability, the Tryon maze-brights and maze-dulls, respectively. (The significance of the correlations between behaviour and the brain acetylcholine system arising from these experiments will be discussed more fully in the second

part of this chapter, which deals specifically with brain acetylcholine, the central nervous system and behaviour).

1.13 Strains and Emotional Reactivity

A further field in which the production of behaviourally selected strains has been profitable concerns behavioural measurement of the effects of stress and levels of emotional reactivity. Experimental psychologists have for many years employed such tests as the open-field test (Hall 1934) to induce mild stress in rats, to measure levels of reactivity or emotionality, as models for human emotionality and also as a useful behavioural measure in behaviour genetics. Studies of the effects of extreme stress have also been used in similar studies. Sawrey and Long (1962) observed strain and sex differences of four strains of rat in susceptibility to stomach ulceration induced by stress. These rats were subjected to a stress situation in which approach to food or water resulted in footshock. Subsequent examination showed sex and strain differences in susceptibility to ulceration. Mikhail and Broadhurst (1965) examined gastric ulcer formation as a result of complete physical restraint in two strains of rat showing different emotionality levels in an open-field test. Failure to detect differences between strains in ulcer formation was thought to demonstrate the existence of several types of emotionality, depending upon type, as well as degree of stress. Sines (1959, 1962) using a physical restraint technique, also examined gastric ulcer susceptibility in a population of rats. By selecting the most susceptible and breeding them together and similarly breeding together the least susceptible for two generations he succeeded in producing two new strains differing in susceptibility to ulceration, confirming the genetic control of this character.

Votava and Souskova (1963) successfully produced strains of rats which differed in their responses to the milder stress situation of, what

was essentially, an open-field situation. They observed the behaviour of rats when given access to a relatively large compartment of a two-compartment box. Exploratory rearing, immobility and preening behaviour were scored and animals showing 'low' or 'high excitability', were selected and bred, then further selected and bred again, producing two new strains. These strains, differing in their reaction to novel surroundings, were also used by these authors to examine the effects of some psychotropic drugs (Votava and Souskova 1963).

By far the most extensive work done on the inheritance of emotionality or reactivity in rats has employed the open-field test of Hall (1934) or a modified form of it (Broadhurst 1957). Hall (1951) measured the responses of rats to the novel and stressfull stimulus of exposure to a large, brightly lit, circular arena, by scoring, in particular, emotional elimination as this response is recognised as a reliable measure of emotional response to stress (Broadhurst 1957). He succeeded in producing by selective breeding, two strains of animals which represented the two extremes of emotional reactivity to the open-field. Broadhurst (1960, 1962) repeated and expanded Hall's work and produced by selective breeding, two more strains, selected for high or low emotionality in a standardised version of the open-field.

These strains produced at the Maudsley Hospital, London, became known as the Maudsley Reactive (MR) and Maudsley Non-Reactive (MNR) strains (Nos. 163 f and 163 g, respectively, in British Laboratory Animal Centre, Catalogue of Uniform Strains). Much work has now been performed with these strains by various workers and something of the nature of their differences may be described. In the open-field the MR strain responds with high defaection scores and low ambulation scores. The MNR strain is much less 'reactive' and defaection scores are low or zero whilst ambulation is usually high (Broadhurst 1960).

The bidirectional selection for high and low defaecation thus also produced a correlated change in ambulation or exploratory activity in these strains. Presumably, the ambulation change arises from the tendency of highly emotional animals, such as the MR strain, to freeze in stressful situations thus inhibiting exploratory behaviour. Because the most obvious difference between these strains is one of emotional reactivity they have been usefully employed in investigations of the part played by emotionality in other type of behaviour. Singh (1959 and 1961) and Singh and Eysenck (1960), examined the behaviour of the Maudsley strains in a conditioned emotional response (CER) test.

In this test rats were trained to press a lever for water reward and then decrements in performance were measured in the presence of a stimulus, which had previously been associated with shock. The MR rats showed high levels of conditioning to the stimulus and large decrements in performance resulted. The MNR strain, however, showed much smaller disruptions. These experiments confirmed the emotionality differences but also show the conditionability of the MR strain to be, apparently, unimpaired by the selection experiments. The conditionability of the MNR strain remained untested because in this experiment it was possible that the lack of a significant response deficit was due to their low level of reactivity. Owen (1962) and later Levine and Broadhurst (1963) and Broadhurst and Levine (1963), found that if the strains were compared in a conditioned avoidance of shock test (a shuttlebox), there were distinct differences in performance. This time the MNR strain demonstrated rapid conditioning but the MR strain was only slowly conditioned, many subjects never attaining high levels of avoidance. Thus in this test, high emotionality was demonstrated to be a disadvantage in avoidance learning. The test required that the subject first made escape responses from shocks by crossing from one side of a box to the other and then made

avoidance responses when presented with a warning stimulus (a buzzer) a few seconds before the shock. The presentation of shocks creates, in an emotional rat, fear responses of crouching, freezing and defaecating, none of which are conducive to the active movement required for conditioning in this test. This explanation is reinforced by the finding (Broadhurst and Levine 1963) that with increasing shock level the avoidance learning of the MR strain becomes worse whilst that of the MNR strain is unaffected. One may assume, therefore, that increasing shock produces increasing fear arousal and this, in turn, reduces the likelihood of avoidance responses being made. Certainly, it does not appear that the MR strain rats are less conditionable because of their CER performance (Singh 1959, 1961), nor less 'intelligent' (Das and Broadhurst 1959), since the strains perform equally well in a Hebb-Williams maze test. Imada (1972) compared the strains for emotionality and conditionability and also found negligible difference in conditionability. In these experiments thirsty animals were allowed to drink in a test box and intermittently given foot shocks through the grid floor. The MR strain showed greater suppression of drinking behaviour than the MNR strain when the shocks were introduced, confirming the emotionality differences. When later, a warning stimulus (light) was presented for 5S before the appearance of each shock, rats began to drink normally again druing the 'no stimulus' phases and only stopped during the 'light/shock' phases. The learning curves for the acquisition of this behaviour were the same for MR and MNR strains showing similar learning potential for the strains.

1.14 Strains and Conditioned Avoidance

Some strain differences in the ability of rats to perform conditioned avoidance behaviour have already been described (e.g. Nakamura and Anderson, 1962; Broadhurst and Levine, 1963 and Owen, 1963). Strain differences suggest an inherited component for this behaviour which may

depend upon levels of emotionality or some other associated factor, or a difference in learning ability per se. To examine this further, selective breeding experiments have been performed to produce strains differing in conditionability and selected on the basis of slow or fast conditioning in a shuttlebox (Bignami, 1965). Bignami, working in Rome, selected the highest avoidance rats from five, fifty-trial training sessions and bred them together and similarly selected the rats with lowest avoidance scores and bred these together. The selection was continued until two widely differing strains were produced which are now known as the Roman High Avoidance Strain (RHA) and Roman Low Avoidance Strain (RLA). A cross fostering experiment was performed with some of these animals to check the role of post-natal effects on their avoidance behaviour. There was no evidence to suggest that the behavioural differences were anything other than genetic. Soon after these strains were produced, Broadhurst collaborated with Bignami and continued the selection and breeding of these strains in England, and began to look for correlative effects of the selection. It will be remembered that the MR and MNR rats differed in conditioned avoidance behaviour and it was suggested by several workers that these differences depended upon their differences in emotional reactivity. Broadhurst and Bignami (1965) began the examination of the Roman strains by comparing their emotionality levels in the open-field. Surprisingly, the strains exhibited similar levels of reactivity when scored for emotional defaecation, thus showing a lack of correlation with the Maudsley strains. There was, however, one similarity with the latter strains; the RHA rats were significantly more active than the RLA strain when measured for ambulation scores in the open-field and when scored for intertrial responses in the shuttlebox. The latter are scored when subjects make shuttlebox crossings between training trials. It has been seen in other work that hyperactive animals tend to learn avoidance responses more

quickly than slow animals. This is particularly noticeable in animals receiving stimulant drugs (Broadhurst and Wallgren, 1964; Buxton, 1972). Holland and Gupta (1966) performed a 'factor analysis' on data obtained from various behavioural measures made on the Roman strains, including open-field behaviour, rearing activity (as a measure of central nervous stimulation or level of behavioural arousal) and spontaneous activity. The indications of their analysis were that the strain differences depended more upon 'activity' components than upon reactivity (emotionality). Imada (1972), however, using the suppression of drinking test described above for the Maudsley strains, suggests that the RLA strain may exhibit greater emotional reactivity than the RHA despite the lack of evidence from open-field tests. The RLA strain show a degree of suppression of drinking in this test, similar to that shown by the MR strain. This occurs at a time when emotional elimination in the test box is zero for the RLA strain. If the suppression of drinking test is a useful means of estimating emotional reactivity in rats, then these results throw doubt on the predictive value of the long accepted open-field test of emotionality. Obviously, the nature of these strain differences are not yet fully understood; the differences in conditionability of the Roman strains may be, in part, due to emotionality differences, but clearly other factors are involved for although these differences do not show up in open-field testing, the differences between MR and MNR strain do.

It is partly the role of this present work to extend behavioural investigations of the Roman strains and to examine other aspects of the differences between them.

1.15 Strains and Psychopharmacology

The use of selectively bred strains in pharmacology has been viewed as a potentially useful approach to drug studies (Broadhurst 1964). Their use has not been extensive but has given some encouraging results. Two approaches have been used. Animals can be bred for a given drug-action so that the phenotype is the differential sensitivity (or insensitivity) to the drug. Examples of this approach may be found in the work of Shaklee and Shaffner (1955) who selected for high and low thyroid response following thiouracil in chickens and radioactive iodine in rats (Sunder 1960). Shagas (1954) selected for sedation threshold in mice to a dose of pentobarbitone and Chase (1950) for high insulin tolerance in mice. By breeding strains specially for the experiments in hand, in this way, it is not only possible to examine the drug response more easily, because of, for example, increased sensitivity, but also the reduction in individual variation presents a more uniform base-line response.

More commonly though, selection experiments have bred for a behavioural characteristic and changes in drug susceptibility have subsequently been studied. Most commonly used in this context have been the Tryon, Maudsley and Roman strains already described. Differential responses of the Maudsley strain rats to drugs have been described by several authors. Easterbrook (1963) trained MR and MNR rats to escape a shock by pressing a panel. He found learning in the MNR strain to be superior to that of the MR strain, paralleling the shuttlebox findings of Levine and Broadhurst (1963) described above. However, when ethanol (0.5 mg/kg.) was administered there was a strain reversal effect, with the MR rats demonstrating the fastest learning speeds (Easterbrook (1963). Presumably, hyperactivity and a reduction in emotional reactivity induced by the ethanol was responsible for the change. Broadhurst and Wallgren (1964) were unable to repeat this effect using conditioned

avoidance in a shuttlebox. Broadhurst (1964), however, demonstrated a similar strain reversal for shuttlebox avoidance learning in the Maudsley strains under the effect of small doses of reserpine. At a dose of reserpine (0.25 mg/kg.) which depressed avoidance learning in the MNR strain there was a significant enhancement of the performance of the MR strain. Holland and Gupta (1966) showed a similar effect ~~*that*~~ with *methyl-pentynol a short acting tranquilliser. Powell, Martin and Kamano (1966) using the Tryon strains, found increases in avoidance responding of the previously low avoidance, high emotionality, S_1 strain after administration of amoborbital. The same dose of the drug produced decreases in avoidance responding when given to the lower emotionality, S_3 strain. These effects can presumably be explained in terms of lessened emotional reactivity brought about by sedatives and tranquillisers, although depression of avoidance behaviour is normally expected with these drugs. Broadhurst, Sinha and Singh (1959) failed to find strain differences between the MR and MNR strains in response to a series of stimulant and depressant drugs when tested in an open field, the situation which provided the criteria for the original selection of the strains. Strain differences in response to nicotine were reported by Garg (1969) who examined the effects of the central nervous system stimulant on rearing behaviour in the Roman and Maudsley strains. Nicotine facilitated rearing behaviour and the RHA and MNR strains were found to be most sensitive to its effects. Doses of nicotine which produced enhanced rearing in these strains produced reductions of rearing of the MR and RLA strains. Bignami (1965), reported an improvement in the conditioning of RLA rats when they were pretreated with d-Amphetamine. In a further attempt to examine the mechanisms underlying the poor avoidance behaviour of the RLA strain, Coyle, Wender and Lipsky (1973) also administered d-Amphentamine and confirmed the improvement. Further examination of their data*

led these authors to conclude that the increase in avoidance conditioning was due to a general increase in activity, seen as increased intertrial responding and not necessarily an increase in specific responding to the conditioned stimulus.

It is one purpose of the present work to look at some aspects of drug action in the Roman strains.

1.2 Cholinergic Mechanisms and the Control of Behaviour

1.21 Cholinergic Mechanisms and the Central Nervous System

It is established beyond reasonable doubt that synaptic transmission in the mammalian nervous system is chemically mediated. Further, it is also established that acetylcholine (ACh) is the neurohumoral transmitter agent at nearly all peripherial nervous system (P.N.S.) synapses, except terminals of the postganglionic sympathetic fibres, where noradrenaline has this function. In addition there is now quite strong evidence for ACh, noradrenaline and dopamine, and some evidence for other substances, being transmitters in the central nervous system (C.N.S.) Because ACh was the first transmitter substance to be identified and probably also because there are many drugs available with which to modify its actions, it has been the subject of much study, both with regard to its role in the C.N.S. and more specifically in the control of behaviour, (see, for example, reviews by Reeves, 1966; Carlton, 1963; Michelson, 1961). So rigorous has been the interest that one author has sought to check the possible 'over exploitation' of the central cholinergic system (Karczmar, 1969). Despite the attention to the ACh system surprisingly little is known definitely of its role, particularly in behavioural control.

ACh in the C.N.S., and the two enzymes responsible for its synthesis and degredation, choline acetyl transferase (choline acetylase, ChA) and acetylcholinesterase (AChE), respectively have been a subject

of study for a long time, an early example is, the work of Feldberg and Vogt (1948). ACh is found throughout the C.N.S. but is considerably more concentrated in brain grey matter where mainly synapses and cell bodies are located, than in white matter which consists mainly of axons (Feldberg and Vogt, 1948). The concentration also varies between brain structures; biochemical assays of homogenates show ACh to be most concentrated in the brain stem and caudate nucleus, least in the cerebellum and intermediate in the cerebral cortex, pons and medulla (Macintosh, 1941; Quastel, 1962).

ACh is synthesised from choline and acetyl coenzyme A in the presence of ChA the distribution of which roughly parallels that of ACh (Quastel, 1962), and in subcellular fractionation studies is shown to be located mainly in the soluble cytoplasmic fraction of neurones (de Robertis, 1963; Whittaker, 1964). It appears that ChA is produced in the soma of the cell and transported to the fibre end-feet along the axon (Eccles, 1961), since cutting the axon leads to a transitory build up of ChA at the side proximal to the cut and a depletion at the distal end. ACh is usually only found in and around nerve end-feet, so it is assumed that its manufacture takes place there. Density gradient centrifugation of isolated nerve endings has been used to locate the end-feet ACh in synaptic vesicles where it appears to be bound prior to use. Eccles (1961) in a series of photomicrograph studies of nerve endings had previously demonstrated the presence of synaptic vesicles and suggested that these might hold stores of ACh. Eccles (1961) postulated that the visicles are sterically related to receptive sites on the inner surface of the presynaptic membrane and depolarisation increases the number of available sites. The vesicles attach to these sites ejecting their contents into the synaptic cleft. ACh, once released, reaches receptor sites of the post synaptic membrane and in sufficient

quantity, brings about depolarisation of the membrane and initiation of an action potential. It is then rapidly hydrolysed by attendant acetylcholinesterase preventing prolonged stimulation of the post synaptic membrane.

Various theories of post synaptic receptor action exist but since no pharmacological receptors of any type have ever been isolated, indirect methods of investigation have been employed. Recent work with a snake venom component, α-bungarotoxin, however, (Lee and Tsung, 1966; Changeux et al, 1970; Miledi et al, 1971), may permit the first steps towards receptor identification. Work by these authors suggests that this material, a protein, acts as a cholinergic-receptor labelling agent, which may eventually permit the isolation of receptors in nervous tissue. Although most theories of receptor action are based almost entirely on data and observations obtained from examination of the P.N.S., it may be assumed, in the absence of evidence to the contrary, that similar mechanisms prevail in the C.N.S.

In attempts to examine the roles of the central cholinergic system much work recently has examined, in detail, the location of ACh and its associated enzymes, in the brain. A profitable technique which has been used (to map cells which are sensitive to ACh) is the highly sophisticated technique of microiontophoresis, with which it is possible to apply drugs to single neurones. The ions of the active substance, in this case, ACh, in aqueas solution, are made to pass out of the tip of a fine glass micropipette by means of a suitably directed current. Neurones sensitive to iontophoretic application of acetylcholine have been found in all regions of the C.N.S. which have been examined so far. Although ACh sensitive cells can be found in many brain areas, the percentage of such cells in a given area is often small. Thus in cerebral cortex, for example, 30% of tested cells are cholinoceptive (Krnjevic and Phillis, 1963) and the majority of these are excited by ACh, inhibi-

tory effects being rare (Randić et al, 1964). A noteable finding, from microiontophoretic work with ACh is that cortical cholinoceptive cells appear to be innervated by cholinergic radiations from the brain stem reticular formation (Krnjević, 1964). Further work on the brain stem has shown that about 50% of the cells in the pons and medulla are cholinoceptive, some being excited and some inhibited by ACh (Bradley , Dhawan and Wolstencroft, 1966).

Response of a nerve fibre to artificially applied ACh shows ACh sensitivity, but not necessarily that this fibre normally responds to endogenous ACh, (i.e., bears ACh receptors), but associated pharmacological tests, application of antiacetylcholine (anti-ACh, i.e., antropine-like) of anticholinesterase agents, may help to confirm ACh as the specific agent for the neurone. An example where thorough analysis has confirmed cholinergic sensitivity of a particular C.N.S. neurone, is in the work of Curtis and Eccles (1958) on Renshaw cells of spinal cord. The effects of applied ACh so closely mimic those of synaptic excitation that it is now generally accepted that the synapse between collaterals of motor axons and Renshaw cells is cholinergic. It has further been found that the action of ACh on this synapse is mimicked by iontophoretically applied nicotine, and thus is characterised as a nicotinic synapse, but the majority of brain cholinoceptive neurones appear to carry muscarinic receptors (Karczmar, 1969).

Work of a different kind also suggests that there are muscarinic synapses in the C.N.S. Experiments with the muscarinic agent, oxotremorine are notable in this context. Oxotremorine when given parenterally produces signs typical of peripheral parasympathetic stimulation, including lachrymation, salivation and miosis, but also signs that are more typical of central stimulation namely, muscle tremor and muscle rigidity. These latter effects remain unchanged when oxotremorine

is given after previous treatment with a quarternary anti-ACh drug, such as atropine methylbromide (George, Haslet and Jenden, 1962). Most drugs, including atropine methylbromide, containing a quaternary ammonium ion do not penetrate the blood brain barrier to enter the C.N.S. after parenteral injection. Atropine methylbromide blocks the salivation, lachrymation and miosis produced by oxotremorine but not the muscular tremor or rigidity, pointing to the central origin of the latter responses. Atropine sulphate, however, which easily passed into the brain, blocks all the toxic signs produced by oxotremorine. This and other evidence (Bebbington and Brimblecombe, 1965) strongly suggest the presence of central muscarinic sites, although, whether oxotremorine stimulates these receptors directly or indirectly by increasing available ACh concentrations (Giarman and Pepeu, 1962; 1964, Crossland and Slater, 1968) has been a subject for debate. Further support for oxotremorine acting directly has come from work done recently by Cox and Tha (1973).

The importance of central muscarinic receptors in the control of behaviour is emphasised by the evidence that muscarinic agents such as oxotremorine and arecoline depress certain types of behaviour. Prodham and Dutta (1970) showed arecoline to reduce the spontaneous activity of rats, an effect which was reversed by hyoscine but not methylhyoscine. The quaternary methiodide of arecoline did not produce these effects and mecamylamine, a ganglion blocking drug, was unable to reverse arecoline induced depression, suggesting that the origin of the effect was central and that nicotinic receptors were not involved. In the same paper authors showed arecoline to depress various types of operant behaviour in rats. Injection of nicotine was shown to produce only transient and variable depression of operant self-stimulation in rats whereas arecoline consistently produced severe depression, suggest-

ing the greater importance of muscarinic receptors in this behaviour (Olds and Domino, 1969).

In order that ACh may function successfully as a synaptic transmitter it is essential that it should be inactivated rapidly, and in particular, within time limits imposed by the response characteristics of the nerve complex concerned. Body fluids and tissues in many parts of the body contain cholinesterases which rapidly hydrolyse ACh into choline and acetic acid. Acetylcholinesterase (AChE, specific or true ChE) is found in neurones, at synapses, neuro-muscular junctions and in several other tissues, including, erythrocytes, thrombocytes and placenta. A second cholinesterase, Butyrocholinesterase (BuChE, non-specific or pseudo-ChE) is also widely distributed, but particularly in glial and other associated cells of the C.N.S. and P.N.S. and in plasma, liver and other organs. Both enzymes are capable of hydrolysing ACh but AChE does so at a faster rate and most pharmacological effects seen of anti-ChE agents are due to inhibition of AChE. Inhibition of BuChE produces no detectable effect on the organism and its function in the body is not known. Knowledge of the cytological distribution of AChE has been increased in recent years largely by the development of techniques of microscopic histochemistry (Koelle, 1963; 1969; Schwarzacher, 1961). Such techniques as these have permitted detailed postulates of cholinergic pathways to be made but there may be some hazards in drawing conclusions from this technique. The histochemical technique demonstrates the presence of AChE in many tissues and Karczmar (1969) has warned that increasing the incubation times beyond those normally used, demonstrates many more tissue locations than are normally recognised and raises questions of validity. Another hazard is that, as blood contains much AChE, sections must not be contaminated with blood during preparation.

Brain areas which show particularly high AChE concentrations include the caudate, thalamic and lenticular nuclei, the amygdala, hippocampus, hypothalamus, cortical areas and direct and diffuse projection pathways to the cortex. Rosenzweig, Krech and Bennett (1958), report that the motor cortex has higher activity than the somatosensory area which, in turn, has higher activity than the visual cortex. AChE activity in rats appears to increase with age, reaching a maximum activity at about 100 days and then declining (Bennett, Krech, Rosenzweig, Karlson, Dye and Ohlander, 1958).

1.22 Behavioural Effects of Manipulating Brain AChE

Investigations of the behavioural effects of reducing AChE activity may be divided roughly into two types. First, there has been extensive work on the effects of reducing brain AChE to a low level, either chronically, as exemplified by the work of Russell (1954, 1958, 1964, 1966), Russell, Watson and Frankenaeuser (1961) and Chippendale, Zawolkow, Russell and Overstreet (1972) or by acute reduction such as in the work of Glow and Richardson (1967), Richardson and Glow (1967) Goldberg, Johnson and Knaak (1965), Goldberg, Johnson, Knaak and Smith (1963), Warburton (1969) and others. Secondly, the work of the team of Rosenzweig, Krech and Bennett, some of which has already been described, in which the effects of small changes in brain AChE were investigated, not with drugs, but by selective breeding experiments (Rosenzweig, Krech and Bennett, 1960) on the one hand and by rearing in environments of varying character on the other. (Rosenzweig, Krech, Bennett and Diamond, 1962 and others).

Russell, Watson and Frankenhaeuser (1961) fed 'Systox', (00-diethyl-s-ethylmercaptoethanol thiophosphate) an organophosphate, anti-ChE insecticide, to rats in their diet so that rats with various levels of AChE inhibition were obtained. These rats were then trained in a conditioned avoidance test which involved jumping onto a platform

to avoid a shock. They were later observed for rates of extinction of this response when the shock stimulus was removed. They found that the acquisition of the response was not affected by AChE inhibition until an inhibition of 76.5% of normal, in the brain was reached. The rate of extinction of the response was shown to be inversely proportional to AChE activity, thus the animals which suffered the greatest enzyme inhibition stopped performing the conditioned response soon after the shock was switched off and sooner than controls. Although acquisition was not affected, differential affects on extinction were seen at levels of inhibition of AChE of up to about 60%, which appears to be a critical level beyond which significant decrements in behaviour appears. Aprison (1962) reported some observations on the relationship between ACh and AChE in caudate nucleus and cortex of rabbit brain under increasing AChE inhibition. It was noted that no increase in free ACh occurred until inhibition reached a critical level of about 60%. Below this level the ACh content rose rapidly, 'indicating the loss of physiological control of the enzyme for its substrate'. This work points to the large 'margin of safety' which appears to exist for AChE in the mammalian C.N.S.

A further interesting feature of the work on chronic ChE inhibition is the tendency for a form of tolerance to appear. Rider, Ellinwood and Coon (1952), demonstrated that daily doses of 'Schardan' (octamethyl pyrophosphoramide)an insecticide, enabled rats to withstand otherwise lethal doses of this anti-ChE. Barnes and Denz (1954) fed 'Schradan' and 'Systox' to rats in the diet and demonstrated physiological tolerance to very high levels of inhibition of AChE. Rats receiving high doses showed obvious signs of poisoning in the first few weeks of the experiment but these signs gradually subsided and the animals appeared normal, even at the end of the experiment, (after twelve weeks) when it was shown that only 7% of normal AChE activity was present. No behavioural tests per se were performed on these animals but development of behavioural

tolerance has been described in chronic inhibition studies by Russell, Overstreet, Cotman, Carson, Doyle, Dalglish and Vasquez (1972). Their results showed that behavioural recovery to pretreatment levels occurred without readjustment of ACh or AChE levels. The observations of these authors have led them to suggest that prolonged exposure to high ACh, leads to a decreased sensitivity of cholinergic receptors. This idea is reinforced by experiments (Chippendale, Zawolko, Russell and Overstreet 1972) in which the anti-ACh drug, hyoscine was given to rats previously made tolerant to high AChE inhibition by chronic treatment with the anti-cholinesterase, diisopropyl-fluorophosphate ('Dyflos'). These animals showed an enhanced response to hypscine compared to controls suggesting that the competition for ACh receptor sites had changed to favour hyoscine. Such a mechanism has also been suggested for peripheral sites by Bitto and Dawson (1970).

Acute studies of AChE inhibition show similar examples of disruption of behaviour when thresholds of 50-60% inhibition are exceeded. Thus Glow and Richardson (1967) observed depression of operant responding for food reinforcement in rats, with a single dose of 'Dyflos', which produced approximately 60% inhibition of AChE and Goldberg, Johnson, Knaak and Smyth (1963) disrupted shock avoidance behaviour with a single injection of a synthetic carbamate which produced 50% inhibition. Injections of atropine but not the quarternary analogue methyl atropine, restored avoidance responding, confirming the involvement of central ACh in this disruption.

Not all the effects of reduced brain AChE activity that have been described consist of behavioural impairment. Russell, Watson and Frankenhaeuser (1961), in their experiment with chronic administration of 'Systox', described a tendency for rats to show increased efficiency

of responding at AChE inhibition levels of less than the critical one of approximately 60%. This biphasic effect seen during progressively reducing AChE activity has been described also by Metz (1958) for the respiratory reflex. Potentiation of respiration was seen as inhibition increased to 60%, above which a decline began. Stimulation of ACh activity in this way has been investigated by administering small doses of anti-ChE substances and measuring changes in behaviour. Whitehouse (1966) describes facilitation of discrimination learning and Cox and Tye, (1973) improved acquisition of a 'position habit' by rats in a Y-maze after very small doses of the anti-ChE agent physostigmine. Injection of anti-ChE agents immediately after or at periods after training, have produced effects which lend support to theories of perseveration and consolidation of learning and memory. Facilitation (Stratton and Petrivonitch (1963) or retrograde amnesia (Deutsch, 1969, 1971) according to dose, time after training or site of injection, may be produced. Stratton and Petrinovitch (1963) gave post-trial injections of physostigmine to the Tryon maze-brights and maze-dulls after maze training. The maze-bright strain rats have a higher ratio of ACh : AChE in brain then the maze dulls and it was shown that consequently, a smaller dose of physostigmine was required to produce optimum learning in the former strain than was required in the latter. Weiner and Deutsch (1968) have shown that under certain conditions injections of 'Dyflos' into the hippocampus can facilitate learning of a visual discrimination task.

Despite the small quantities of anti-ChE agents employed in this group of experiments the AChE inhibition was probably within the range of 20-50%; the effects of much smaller changes from 'normal' ACh : AChE ratios have been investigated by means of genetic manipulations and changes in the 'complexity' of the environment.

The breeding experiments performed by Rosenzweig, Krech and

Bennett (1960) have been described above as also have the ACh : AChE measurements performed on the brains of rats from these and other strains. These authors have attempted to correlate small brain differences in these chemicals with behaviour, in particular, 'adaptive' maze learning ability ('hypothesis behaviour'). Their original work with the Tryon S_1 *and* S_3 *strains showed that 'maze-brightness' was associated with higher than normal AChE activity and ACh level which the authors suggested may provide 'more efficient neural transmission' permitting better 'adaptive behaviour'. These conclusions may overlook, however, the observations of other workers which have shown that the learning ability of these strains differ only in certain test situations, Searle (1949) and McGaugh, Jennings and Thomson (1962). There is thus a danger in interpreting these findings too widely. The latter authors discovered that if the Tryon rats were trained with various intervals between trials, instead of massing trials, as had been done previously, it was possible to show differences in consolidation rates for the strains. Possibly this finding is relevant to ideas of cholinergic involvement in consolidation theories of learning and memory.*

Later experiments carried out by Krech, Rosenzweig and Bennett (1960, 1962) and Rosenzweig (1966), investigated the effects of various environments on several brain functions. They divided litter mates between several contrasting types of cage surroundings so that adequate control was provided for any changes that might be produced. The environments were of three types, described by the authors as varying from 'environmental complexity' to 'environmental impoverishment'. The former situation entailed housing the test animals in groups of twelve in cages containing many objects, (referred to by the authors as 'toys') and giving access each day to a maze for exploration. Some training in maze performance was also given each day. The second condition was referred to as 'social control' and consisted of a normal home cage with

cage mates, whilst the 'impoverished' conditioned was social isolation in a cage with opaque sides and no opportunity for mixing with other rats or apparatus. Subsequent examination of the brains of these animals revealed several surprising features. It was found that compared with rats raised in the 'poor' environment, those raised in the 'rich' environment had heavier visual and whole cortices, a greater proliferation of glial cells; an increased activity of AChE and BuChE in the brain, a decreased ratio of the activities of cortical to subcortical AChE and also an increased concentration of brain ACh. The authors were able to demonstrate that the animals reared in the 'rich' environment were significantly superior to the 'poor' environment rats when compared for learning ability in certain types of adaptive learning tests. The 'social control' group fell between the extreme groups in brain parameters and in learning ability. It is difficult to assess the significance of these findings in the context of other work on the role of brain cholinergic mechanisms and behaviour. The differences in ChE activities between the extreme types were small. For example, the total cortical difference was found to be 3% and the sub-cortical 2%. The differences in BuChE in the cortex was 9% and sub-cortically was 1%. It was suggested that these changes may reflect a change in the number of cholinergic synapses. The changes in brain ACh induced by other workers using drugs to produce behavioural change, are very much greater than those observed here and it is difficult to see how the two types of discovery can be compatible. As several changes in chemistry and anatomy were found by these authors perhaps other changes, so far undetected, may exist which are responsible for the behavioural differences. The size of the AChE differences are so small that when it is considered that the samples were taken from a relatively large brain area, encompassing different functional zones, their importance may be seen to be questionable. It was concluded by

Chow and John (1958), after finding that anti-ChE drugs given to rats failed to impair adaptive behaviour in a maze, that 'hypothesis behaviour is not dependant on the levels of .... ChE's or ACh'. In the latter experiments enzyme inhibition was profound.

1.23 The Behavioural Effects of Anti-ACh Drugs

The observation that anti-ACh drugs are capable of modifying behaviour was made a long time ago after experience with the naturally occurring alkaloids, atropine and hyoscine. The behavioural effects of these substances in man were described by de Boor (1958). There has been some doubt about the relationship between these behaviour changes and anti-ACh activity, since Abood (1959) examined a series of synthetic compounds for anti-ACh activity and ability to produce behavioural changes. He compared peripheral anti-ACh activity with behavioural change, which may explain the lack of correlation that he found. He was, however, unable to antagonise the behavioural changes with anti-AChE agents, but Forrer and Miller (1958) and Albanus (1970) were successful in reversing,with phyostigmine,behavioural changes due to atropine.

Several authors have examined the effects on brain ACh of administering anti-ACh drugs. Crossland and Slater (1968) showed a decrease in bound ACh in whole rat brain after atropine (25 mg/kg.). Pazzagli and Pepeu (1964) showed that rats injected with hyoscine (0.5 mg/kg.) shortly after maze-training, showed degrees of retrograde anmesia proportional to the percentage decrease in brain ACh. The amnesia in this experiment had the same time course as the ACh reduction and it was found that although the amnesia could be antagonised by physostigmine or amphetamine only the former increased ACh levels.

One of the most striking effects of most anti-ACh drugs on rats is the hyperactivity produced; Abood (1968) used this property to assess a group of anti-ACh glycollate esters and rank them for potency. This

effect sometimes complicates the interpretation of behavioural testing of anti-ACh drugs. Hyperactivity and enhanced exploratory activity can predispose subjects to faster learning in some types of test, the effect need not necessarily be specific for anti-ACh drugs. It is thus more impressive if the result of giving an anti-ACh drug in an active avoidance or maze test situation, is one of decreased performance. Alternatively, the use of post-trial injections where possible, may rule out this complication. Some authors have found no effects of anti-ACh drugs in discrete trial avoidance, including, Neimegeers (1962) using a shelf-jump test with rats and Pfeiffer and Jenney (1957) using a pole-jump test. In each case the drug, atropine, was given before training. Bignami, Robustelli, Janku and Bovet (1965) measured enhanced performance in a conditioned avoidance test (shuttlebox), after giving the anti-ACh drugs Benactyzine and Ditran, and Brimblecombe and Buxton (1972) also observed enhanced shuttlebox learning produced by some synthetic anti-ACh drugs. To balance these findings, Burosova, Bures, Bohdanecky and Weiss (1964) using a one-trial passive avoidance test showed disruption of acquisition after atropine administration.

Many of these apparent differences in the actions of anti-ACh drugs on behaviour can possibly be attributed to differences in dose, timings of treatments and type of test used.

1.24 The Interaction of the Central Cholinergic System with Other Central Systems

Carlton (1962), when examining the effects of atropine on rats in a non-discriminated (Sidman) avoidance test, (i.e., shock avoidance without the use of a conditioned stimulus) interpreted the disruption seen to a loss of inhibition. The rats exhibited behaviour that had only been exhibited in the early stages of training and which had been lost as the correct responses were learned. Carlton later hypothesised

(1963) that atropine blocked a central cholinergic inhibitory system and assumed the presence, therefore, of a second system which activated behaviour. Thus a tendency of an animal to respond would be positively related to the level of activation and inversely related to the activity of an antagonistic cholinergic system. The idea was not entirely new, since there were many suggestions in the literature previously and have been many since, which have suggested central antagonistic systems. Most of the evidence suggests that the activating system is adrenergic and thus may be stimulated by amphetamine. Tripod (1952) and Galambos, Pfeifer, Gyorgy and Molnar (1967) showed that amphetamine-induced hyperactivity in mice was enhanced by administration of atropine or benactyzine but inhibited by the cholinergic agent, tremorine. Physostigmine was found to antagonise the arousing effect of amphetamine on barbiturate induced sleep in mice (Barnes and Meyers, 1964), whilst amphetamine restored atropine-impaired maze-running in rats, (Michelson, 1961). Anti-ACh drugs have been reported to increase the toxicity of amphetamine including, an increase in the 'social toxicity' of amphetamine in mice whilst eserine and tremorine reduce its toxicity (Mennear, 1965; Morpurgo and Theobald, 1964). The behavioural disturbances seen in man after the anti-ACh drug, parpanit, were prevented by previous administration of amphetamine (Michelson, 1961). Schelkunov (1967) reviews several other examples and suggests a role for such interactions in psychotropic drug action.

The existence of interacting dopaminergic and cholinergic systems in the C.N.S. has also been suggested. Scheel-Kruger (1970) and Arnfred and Randrup (1968) suggested such an interaction after work on dopaminergic sterotyped behaviour in rats. Apomorphine-induced gnawing behaviour was enhanced by amphetamine but inhibited by physostigmine and tremorine. Klawans (1968) suggested that Parkinsonism may result from

an inbalance of such a dopaminergic-cholinergic system. (See also in this context, the review by Hornykeiwicz, 1966).

Carlton (1963) gave rats trained in an operant, Sidman avoidance response a sub-threshold (for behavioural change) dose of atropine followed by amphetamine and noted an enhanced performance over amphetamine alone. Stein and Seifter (1960) gave small doses of reserpine which reduced response rates in a similar test situation and found that small doses of amphetamine restored the operant behaviour whilst atropine produced only slight improvement. Presumably, amphetamine caused reactivation of the adrenergic system, after catecholamine depletion by reserpine, had produced behavioural depression, but atropine block of the cholinergic inhibitory system would cause little activation in the absence of effective catecholamine concentration. In a different experiment, Tripod, (1952) gave doses of amphetamine and atropine chosen to produce equal rises in activity in mice and then gave reserpine to depress activity. It was found that a larger dose of reserpine was required to depress amphetamine-induced increases than atropine-induced increases.

A further extension of the role of an inhibitory cholinergic system suggested by Carlton (1963), arises from the observation (Hearst, 1959), that atropine produces 'perserveration' of responses. That is, a rat tends to repeat responses more readily under the effects of atropine, even after the 'need' to make the responses is gone. Thus rats trained to alternate lever responses between two levers, tend to repeat responses on one lever and extinction was prolonged or even blocked. Carlton (1963), then, suggested that the cholinergic inhibitory system inhibits responses which are not reinforced and thus provides a basis for discrimination in learning. Thus the level of activation controls the tendency for all responses to occur and the cholinergic system acts to antagonise this action on non-reinforced ones. Carlton (1968) developed the idea of the inhibition of non-reinforced responses to explain

the phenomenon of habituation.

Few attempts have been made to localise such a system in the brain but recently Aprison, Kariya, Kingtgen and Toru (1968) measured various chemicals, including ACh, in certain brain areas of rats and found changes in ACh levels in the telecephalon which corresponded with periods of behavioural excitation. Warburton (1969) suggested that the hippocampus might be the site of action.

There is evidence for a differential rate of development of cholinergic and adrenergic systems in the brains of young animals. (Feighly, Parsons, Hamilton and Spear, 1972). These workers found that whilst amphetamine increased activity of ten day old rats atropine produced no effect on activity until twenty days of age. It was subsequently shown that habituation could not be detected in rats until twenty days of age.

There are a few examples in which cholinergic stimulation combined with adrenergic stimulation has not resulted in an enhanced response (Pazzagli and Pepeu, 1964; Linuchev, 1961), but nonetheless, the accumulated evidence seems strongly to suggest that a system involving an activating system which may be adrenergic, but may also include a dopaminergic component, and an inhibitory one which is cholinergic, exists. This system, although undoubtedly oversimplified by this account, may play an important part in the control of certain aspects of behaviour and in particular, learning.

1.3 Aims of the Present Work

The strains of rat chosen for use in this work were the Roman High and the Roman Low Avoidance strains, with Porton strain animals serving as a control or reference strain. The main aim of the work presented in this thesis was to use these animals, which display distinct behavioural differences, to investigate the role of central cholinergic mechanisms in the control of behaviour. It was also hoped that as a result of this work some assessment might be made of the potential usefulness of selected strains of rat, such as the Roman strains, in pharmacological research.

The means by which the ends were pursued are stated briefly as follows:

(a) Observation and analysis of behavioural changes occurring in the three strains after the administration of drugs known to modify cholinergic activity. The behavioural parameters measured were of two types; first spontaneous locomotor activity, including exploratory activity, and second, conditioned avoidance behaviour using a shuttle-box. The drugs used were also of two main types: first, two drugs which antagonise ACh at muscarinic sites, N-ethyl-3-piperidyl benzilate (NEPB, structure shown in Figure 1-1), which has been shown to have both peripheral and central effects (Brimblecombe and Green, 1968) and the quaternary version of this compound, N-ethyl-3-piperidyl benzilate methiodide, which, it is predicted, will have only peripheral anti-ACh activity. Secondly, two drugs which reversibly inhibit the enzyme AChE; physostigmine which inhibits AChE in the peripheral and central nervous systems and pyridostigmine, a drug of approximately similar potency but which, it is thought, acts, in vivo, mainly on peripheral AChE.

(b) Observation of behavioural changes after pharmacological manipulation of central adrenergic mechanisms, which are thought to be

involved in the control of some aspects of behaviour and further, believed by some (Carlton, 1963) to be closely inter-related with the cholinergic system.

(c) Examination of the effects of the same drugs on ACh concentration in whole brain, and in particular, the ACh in specific areas of the brain. The doses used in these experiments corresponded, as closely as possible, to the doses used in the behavioural experiments.

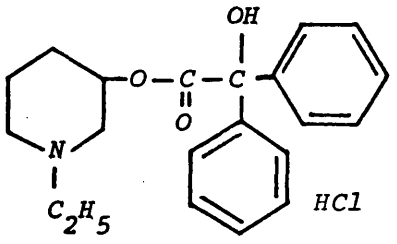


Figure 1-1.

CHAPTER 2

Choice of Behavioural Tests and Experimental Materials

2.1 Behavioural Tests

The Roman strains of rat chosen for use in these experiments demonstrate opposite extremes of conditioned avoidance behaviour in a shuttlebox, and as it was hoped that these differences might be conveniently exploited in this work it was essential that a test of conditioned avoidance, and in particular, a shuttlebox, should be used.

The shuttlebox test of conditioned avoidance behaviour (first described by Warner 1932), is one of many tests that have been used by experimental psychologists and pharmacologists to measure conditioned behaviour in laboratory animals. Tests of this type involve training subjects to avoid a mildly unpleasant stimulus (usually a small electric shock). This stimulus is referred to as an unconditioned stimulus because the subject is expected to make the appropriate escape response (the unconditioned response) without the need for practice. This response in a shuttlebox, consists of crossing from one side of a rectangular cage to the other, by jumping a barrier or passing through a door in a dividing partition. Shock is applied to the grid floor of the cage as the unconditioned stimulus. The following response consists of running back into the first compartment again to escape a further shock and so on. A second stimulus, often a light or buzzer, initially, of no behavioural consequence to the animal, but, is paired repetitively, with the unconditioned stimulus (usually immediately before it), and takes on an important new function as the conditioned stimulus. Conditioned avoidance is demonstrated when presentation of the conditioned stimulus to the animal elicits a response which resembles that made to the unconditioned stimulus. Such a response prevents

the onset of the shock.

Several ingenious variations of conditioned avoidance tests have been used, for example, Domino and Caldwell (1965), described a 'pole-jump' test for rats in which the avoidance response consisted of clinging to a pole suspended from the test-cage roof to avoid an electric shock applied to the grid floor. A 'shelf-jump' test has been reported (Herz 1960) in which shock to the grid floor is avoided when the subject jumps onto a platform or shelf mounted on the cage wall. Various types of operant conditioning, in which a response such as lever, or panel pressing enables the subject to avoid a shock have been used, although strictly speaking, an operant such as lever pressing, cannot be regarded as a truly unconditioned response.

A shuttlebox is, in many ways, a convenient method for measuring conditioned avoidance behaviour. Rats learn the response quickly so that the test may be used to examine the effects of drugs on acquisition of the response as well as on the learned response. Some tests of conditioned avoidance, especially operant behaviour, require lengthy training periods and the subjects cannot be used until trained to a consistent level of performance. A criticism, however, of the most commonly used form of the shuttlebox test, 'two-way avoidance' as used here, is that the conditioning may be delayed, or impaired, because on successive training trials the animal is required to return to a compartment of the box in which it has recently received a shock. Rats tend, normally, to show a passive avoidance of such situations and so may demonstrate, what may be described as 'conflict-behaviour' in the early stages of training, which may slow the learning of the required response. 'One-way avoidance' has been used to lessen this effect; here the animal is only shocked on one side of the box and always escapes or avoids into the other. After each trial the animal is replaced, manually, into the

shock-side, for the next one. The effects of frequent handling throughout the training, however, may be as undesirable as the conflict states of 'two-way avoidance'.

One other aspect of behaviour was chosen for study in this work and this was the measurement of spontaneous locomotor activity. Many different components of spontaneous activity of animals are recognised and the activity measured is very dependent on the conditions present during the recording and the mechanisms employed to make the recording. Small laboratory animals, such as rats and mice, when introduced into new surroundings, spend a considerable amount of time investigating them. This constitutes what is often referred to as exploratory activity and is usually a compound measure of exploration and locomotor activity. Test situations have been devised to measure exploratory activity in small mammals, and include such tests as, 'tunnel-boards' and 'hole-boards' (Steinberg 1964)which permit some quantitation of this activity, Robbins and Iverson (1973) used a technique which purported to measure simultaneously, locomotor activity and exploration as independent measures. Many devices have been used to measure spontaneous locomotor activity in small animals and possibly almost as many types of activity may be measured as test methods that are available, so dependent is the activity on the conditions present. Thus activity has been measured by placing mice into 'jiggle-cages', small boxes suspended from springs which transmit movements to a sensitive lever so that they may be counted. Dews (1953) used a photocell cage, which has since become popular in many laboratories, and activity wheels have also been much used. Many devices depend upon the animals' feet making electrical contact with metal plates or bars in the cage floor. Such techniques require that small currents pass through the animal when connections are made and it is imperative that these small shocks should be well below the threshold

of feeling for the subject, but large enough to reliably record over a wide range of electrical impedances. Another much used technique records movements of small animals by counting the number of times subjects pass between, and thereby interfere with, electromagnetic fields set up in the cage.

Some of these methods provide physical feed-back to the animal for each of its movements, which may influence the activity subject. For instance, the jiggle-cage and activity-wheel, both of which, through their own inertia provide a small stimulus to the animal each time it stops moving. Photocell cages, and most of the electrical devices mentioned, record undisturbed activity, although not all record the same components of it. Thus photocell cages will only record movements that occur where the light beams fall and many of the electrical devices have spatially limited sensitivity ranges. For this work a device was chosen which provided a record of walking activity of, an apparently, undisturbed nature, dependent upon the subject completing electrical circuits with the feet across steel bars of the cage floor, (a full description is given at the beginning of Chapter 4).

When animals are placed into such an activity cage and records of activity taken at short time intervals, an initial high activity period is seen which rapidly falls away to a low level, steady activity phase. The first phase of activity represents the period during which the animal explores the cage and for the purpose of this work will be referred to as the exploratory period, whilst it is not strictly possible to distinguish between exploratory and non-exploratory components in this period, it may be a fair measure because a very large component of this early period is exploratory. The second period of activity will be referred to as locomotor activity and assumed to have little or no exploratory components contained in it.

2.2 The Choice of Drugs

The drugs used in this work were as follows: physostigmine salicylate, pyridostigmine hydrobromide, N-ethyl-3-piperidyl benzilate hydrochloride (NEPB), N-ethyl-3-piperidyl benzilate methiodide (NEPB MeI) and d-Amphetamine sulphate. All drugs were dissolved in physiological saline (0.9%) and injected in volumes of 1.0 ml./kg. Physostigmine, pyridostigmine and d-Amphetamine were injected subcutaneously (s.c.) into a hind-limb, NEPB and NEPB MeI were injected intraperitoneally (i.p.). The anti-ACh drugs were injected by the i.p. routes because in some previous work by this author it was observed that s.c. injection of atropine-like drugs, to the leg of rats produced a temporary paralysis of the injected limb. Such an effect was clearly undesirable and consequently, i.p. injections were favoured for these drugs in this work.

Fresh drug solutions were prepared for each experiment.

2.3 Animal Housing and Handling

All the animals used in this work were bred and maintained at this establishment. The Porton strain rat used in these experiments is maintained by random breeding and was originally derived from a Wistar stock. The Roman strain rats were also produced by random breeding within strains. Male and female animals of all strains were used as specified in the text and were used in the weight range 180-240 g. They were housed in groups of twelve in translucent, white plastic cages with loose wood shavings as bedding. They received food and water ad libitum and were exposed to a 12h light/12h dark cycle. Temperature in the stock-room was maintained as far as possible at 70°C. The temperature did not fall below this level but in hot weather occasionally rose to approximately 78°C. After arrival at the laboratory all animals were housed in the stock-room for a period of

not less than seven days before use in an experiment. Testing was carried out in rooms adjacent to the stock-room and animal handling and transporting after arrival at the laboratory was kept to a minimum. Animals on arrival were randomised into test groups and given coloured tail stripes for identification. Transport between home cages and test chambers was in specially constructed carrying boxes (the size of shoe-boxes) with 'Tufnol' sides and base and transparent, ventilated lid. Handling, except during drug injections or marking, was limited to picking up by the base of the tail, a method believed to reduce trauma to rats that were little used to handling. Animals that were accidently traumatised (e.g. dropped) were removed from the experiment.

CHAPTER 3

Conditioned Avoidance Experiments

3.1 Materials and Procedure

All the conditioned avoidance experiments were conducted using an automatic shuttlebox (Ugo Basile, Milan) (Figure 3-1). The apparatus consisted of a conditioning cage, 47 cm x 20 cm x 20 cm with walls of opaque, grey plastic, a transparent perspex lid and stainless steel grid floor. The grid floor bars were 3 mm in diameter and spaced 10 mm apart. A partitioning wall of black-painted wood divided the cage into two equal parts and a small hole in the partition allowed movements of the rat between compartments. The connecting door remained open throughout the experiments, permitting subjects to cross freely at any time.

A programming unit provided sequences of conditioned stimuli (CS), a light (12w, 250v) mounted in the centre of the lid of the cage and a tone from a small loud-speaker mounted in the centre of one cage wall. The grid floor could be supplied with scrambled shock as the unconditioned stimulus (UCS) and was mounted about a central pivot allowing a see-saw action (with small movement) such that crossings of the rat from side to side produced motion in the grid and moved a magnet over a reed switch on the cage frame, thus permitting crossings by the rat to be recorded.

A training trial consisted of 3s of tone and light together (CS), followed immediately by 4s of shock to the grid with the continued presence of the CS. Three types of response to these stimuli were recognised; the response of running from one side of the box to the other during the presentation of shock (UCS) was termed an escape and this action terminated the remaining portion of CS and UCS.

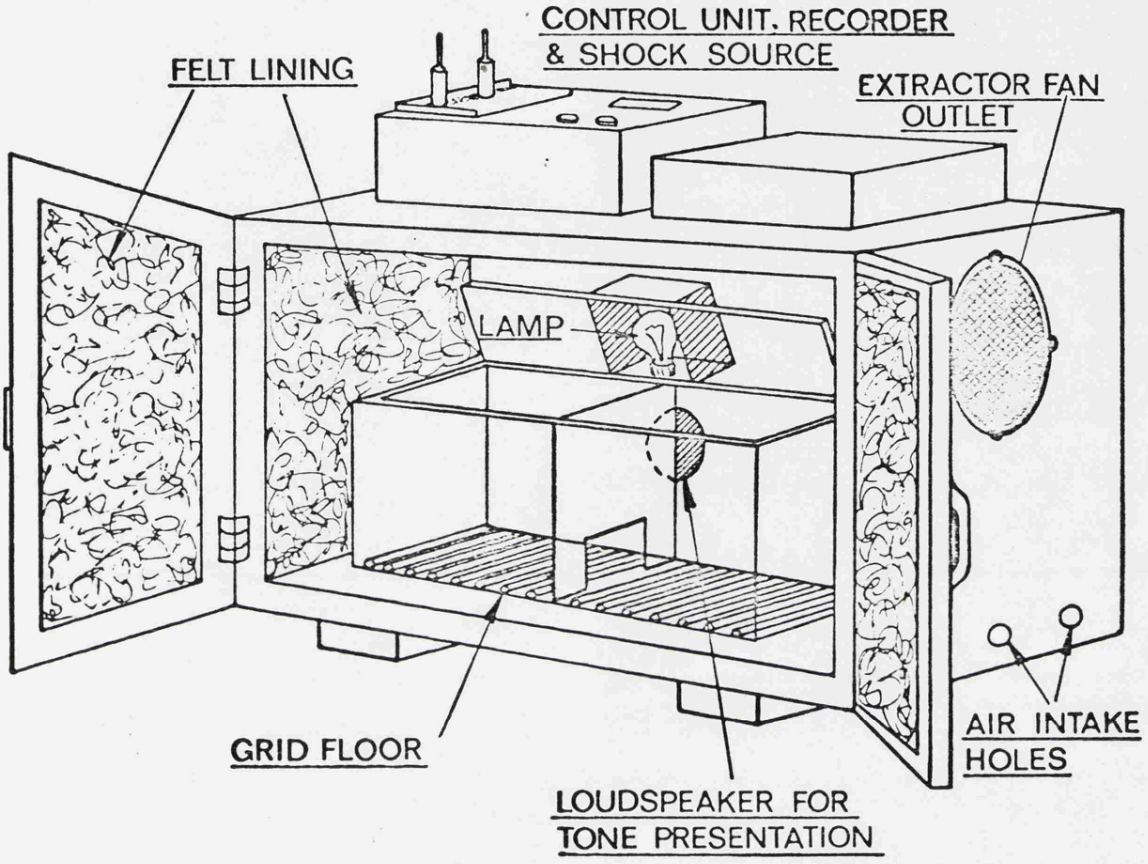


Figure 3-1. Diagrammatic representation of the shuttlebox and sound-attenuating cupboard. Details of the shuttlebox, including its dimensions, are given in the text.

A response occurring during the presentation of CS alone was termed an avoidance and this terminated the remaining portion of the CS and prevented the onset of the UCS. A complete failure to make a crossing response during presentation of CS and UCS was termed a failure.

The shock level was set to give approximately 0.5 mA current to the feet of the rat, a level previously found to consistently produce escape movements in normal Porton rats without apparently causing excessive pain. A previous experiment to investigate the relationship between shock level and avoidance learning in the Porton rat (work by this author, unpublished), had shown this shock level to be suitable for use in this shuttlebox. The relationship between shock level and avoidance learning is shown in Figure 3-2.

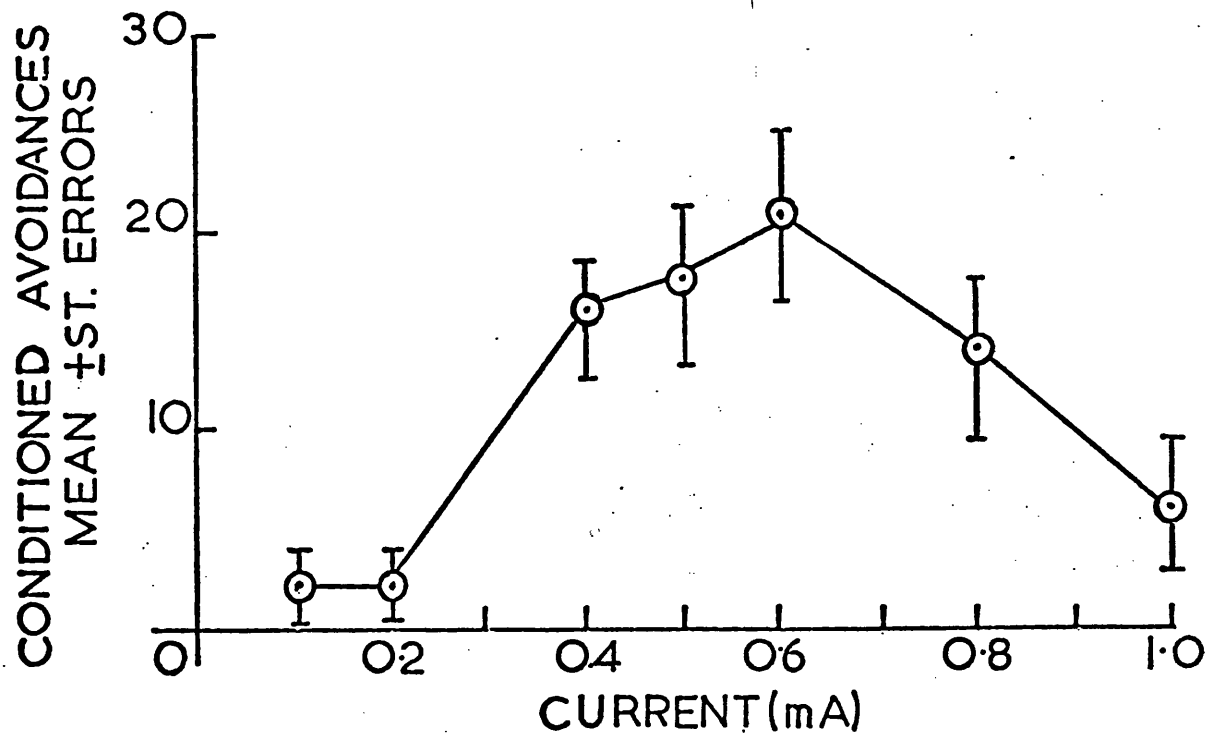


Figure 3-2. Shuttlebox avoidance learning at different levels of shock stimulus (Porton male rats, 8 animals trained at each shock level).

It is apparent when observing rats in this test that shock levels of 0.7 mA and above cause excessive pain, leading to violent escape attempts by jumping at the cage lid and walls instead of running across to the other side. This level of shock thus, actually leads to poorer avoidance than that produced by lower levels. Moyer and Korn (1964) performed a similar experiment with a rat shuttlebox and found optimal avoidance learning with shock levels between 0.5 and 1.0 mA.

The level of shock actually received by an animal at any given moment is difficult to assess and depends upon several factors. The arrangement of the feet on the bars decides the amount of body tissue between any two terminals and therefore the electrical resistance presented. Dryness of the feet increases the skin resistance (especially noticeable when anticholinergic drugs have been administered) with subsequent reduction in current received. Urine on the bars produces an appreciable rise in conductivity and subsequent increase in current received. Figures quoted for shock levels used in these and other experiments, employing this method of shock application, should therefore be considered advisedly.

Training trials were presented at the rate of two trials per minute with constant intertrial length. It was not possible to randomise intertrial length with this equipment, but 'spot check' observations during training sessions provided no evidence for complications due to anticipatory responses. Training sessions consisted of fifty consecutive trials. Animals were allowed five minutes in the shuttlebox at the beginning of each training session to explore the box and settle down after handling.

The shuttlecage was housed inside a sound-attenuating cupboard made of wood, lined with heavy felt and containing a houselight (15w, 250v) and a mains operated extractor fan providing venti-

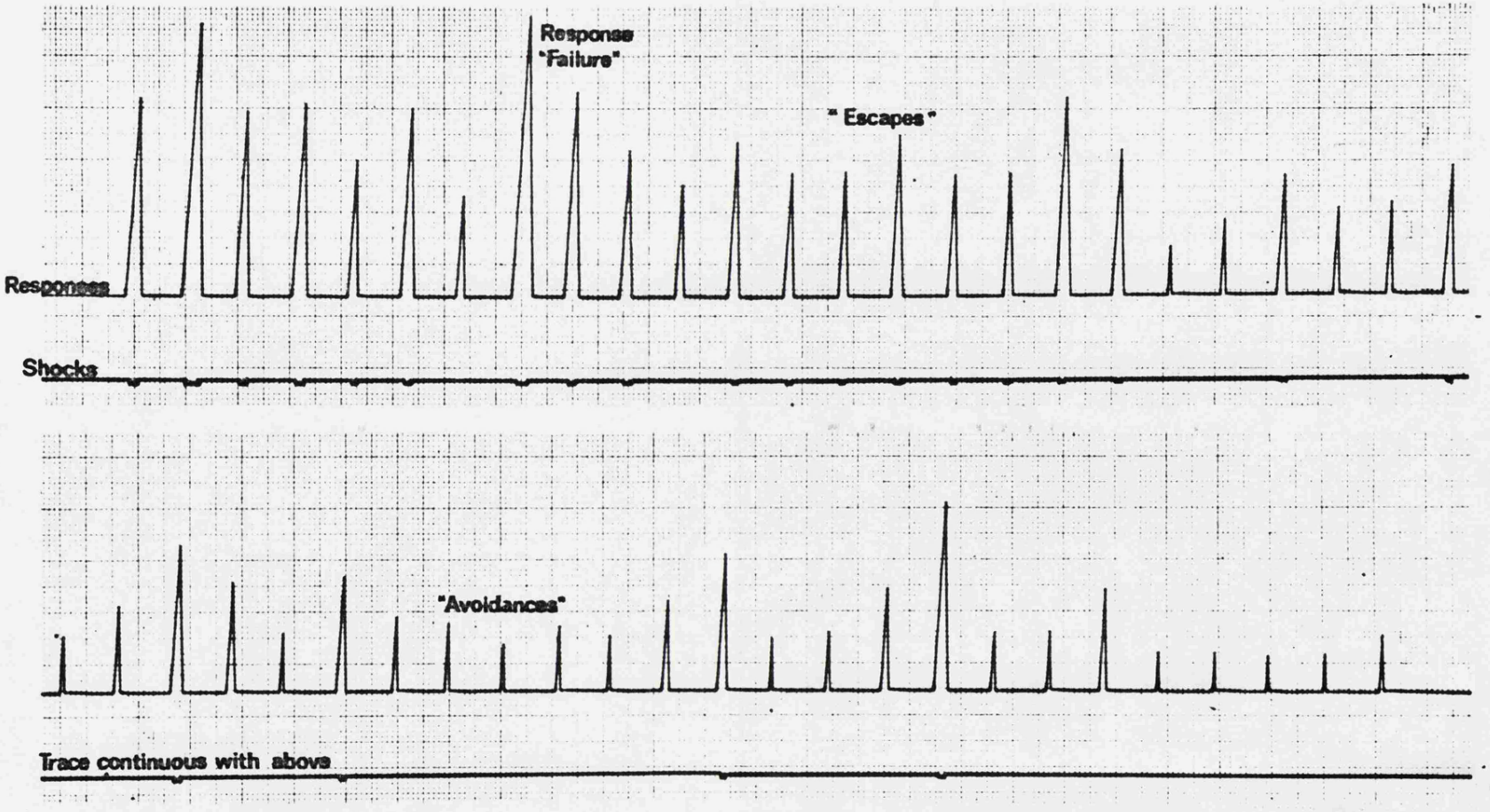


Figure 3-3. A typical shuttlebox pen-recording.

lation and low level background noise. The programming unit and recorder were almost silent in operation, and were mounted outside the cupboard.

The control unit provided a pen recording of responses as a series of 'spikes', the heights of which were proportional to the 'response latencies'. The response latency as measured, was the time elapsing between the onset of CS and the response of the animal. A second pen recorded a 'pip', when shock was applied and it was thus possible to distinguish, on the trace, responses which were escapes and those which were avoidances. A failure to perform either was seen as a maximum excursion of the first pen across the paper. An example record is shown in Figure 3-3. At the completion of an experiment the total number of avoidances and failures for each animal was computed and the data expressed as means with standard errors, for each test group at each session.

3.2 Normal Conditioned Avoidance Behaviour of the Strains

Procedure

The acquisition of conditioned avoidance behaviour was examined in both sexes of the three strains of rat. Subjects were 10 males and 10 females from each of the Porton, RHA and RLA strains randomly chosen from stock. Each subject was given one 50 trial training session per day on 5 consecutive days. On each occasion, the subject was placed into the shuttlebox and allowed 5 minutes in which to recover from handling and to explore the box before the first trial began. One minute after the final trial had finished the rat was lifted from the box and returned to its home cage. The cage bars were then wiped clean before introducing the next subject. Care was taken to ensure that each animal was trained at approximately the same time of day for each of its training session and as only one shuttlebox was available, male and female

rats were trained alternately through the day, in order to avoid effects that might be due to circadian variations in behaviour. This precaution was considered necessary, because a 24 hour rhythm of avoidance learning behaviour has been demonstrated (Davies, Navaratnam and Redfern, 1973).

Results

Avoidance learning in the three strains is shown in Figure 3-4, Porton strain, Figure 3-5, RHA strain and Figure 3-6, RLA strain. The figures show avoidance learning through 5 consecutive training sessions. The RHA strain rats of both sexes, showed a high conditioning speed and reached higher levels of avoidance than either of the other two strains.

The RLA strain, however, showed very low performance in the shuttlebox and avoidance conditioning was not significantly different from zero. Figure 3-6, therefore, shows the escape behaviour of this strain to shock which will be used as a measure of unconditioned responding. The graph shows the number of times shock presentation was not followed by an escape response. Very poor responding was seen, even on this parameter of behaviour, and there was a tendency for these animals to perform more badly with successive sessions. The Porton strain rats (Figure 3-4) showed, by comparison, high rate of avoidance conditioning, but one which was poorer than the RHA strain and the final levels of conditioning were less than those attained by the RHA strain.

A consistent finding through these experiments was that female rats attained greater numbers of conditioned avoidances than males. This difference between the sexes was significant at one training session in each of the strain experiments and the overall difference was most pronounced in the RHA strain. Here the number of conditioned avoidances gained by the female rats was consistently greater than those gained by the males at each of the training sessions although only one these differences reached significance.

The performance of both male and female RLA rats was consistently

poor but, the female rats tended to show greater numbers of escape responses than the males and in one training session this was a significant difference.

Although female rats of all strains tended to show more efficient conditioned avoidance responding than the males and might therefore be considered more useful experimental material for this work, it was decided to use males for two reasons; first, females tend to show variations in behaviour, caused by oestrous cycle changes, (variations in spontaneous activity have been seen, this author, unpublished observations) and it was considered undesirable to introduce unnecessary variation into these experiments. Secondly, the supply of animals for these experiments was such that males were usually available from stock, in greater numbers than females.

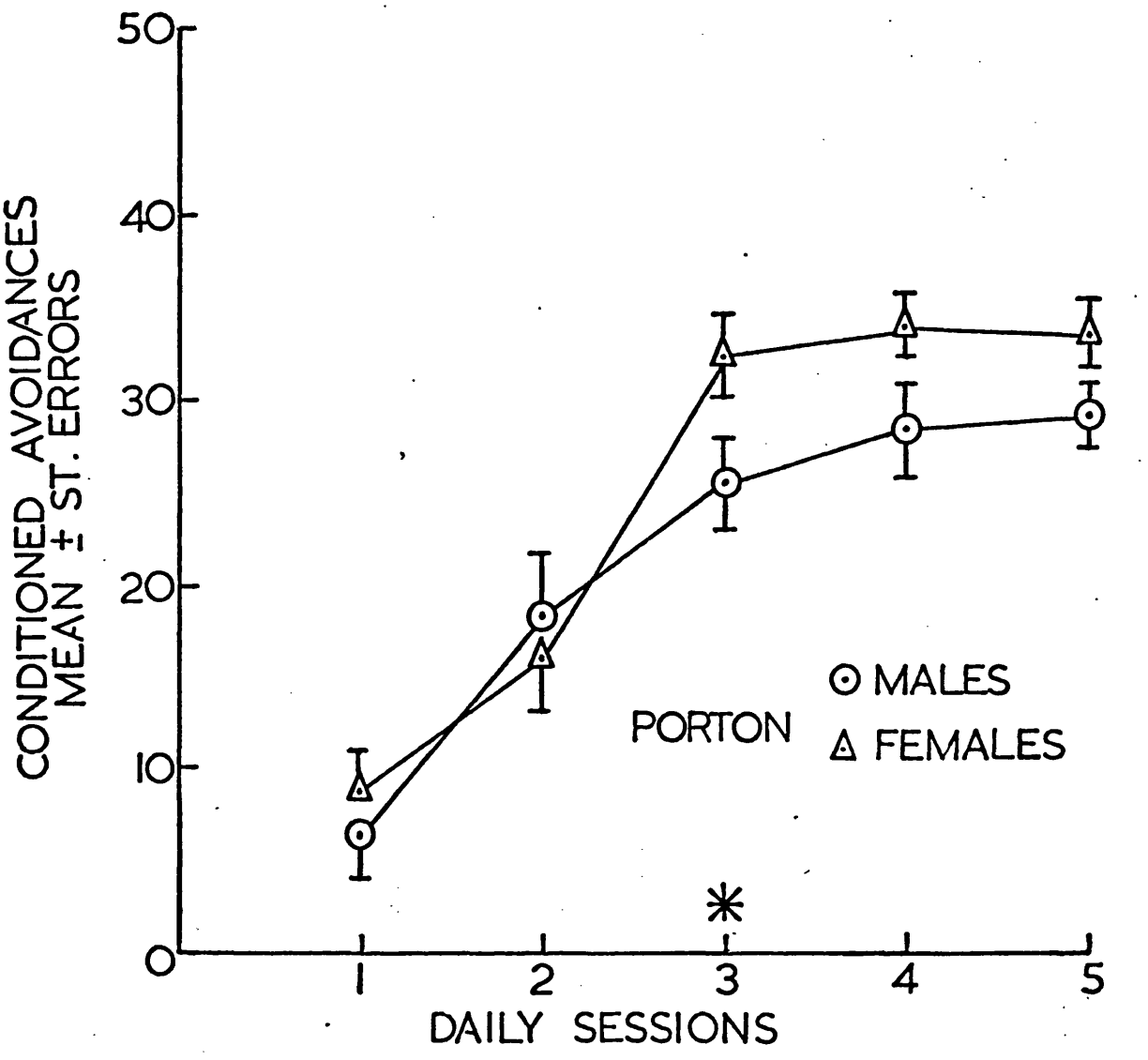


*Figure 3-4. Avoidance learning in males and females of the Porton strain rat. The points represent the mean number of avoidance responses obtained, out of a maximum possible of 50, on successive days of training by groups of 12 rats. Standard errors about the means are shown as vertical bars. Standard errors are only shown on both sides of the mean when space permits, i.e., when the standard error about a mean does not overlap the mean of the other group. When standard errors only, overlap they are slightly offset to permit maximum representation. This convention will be followed whenever means and standard errors are shown in graphical form. * represents a significant difference between means when compared by Student's t-test ($P < 0.05$).*

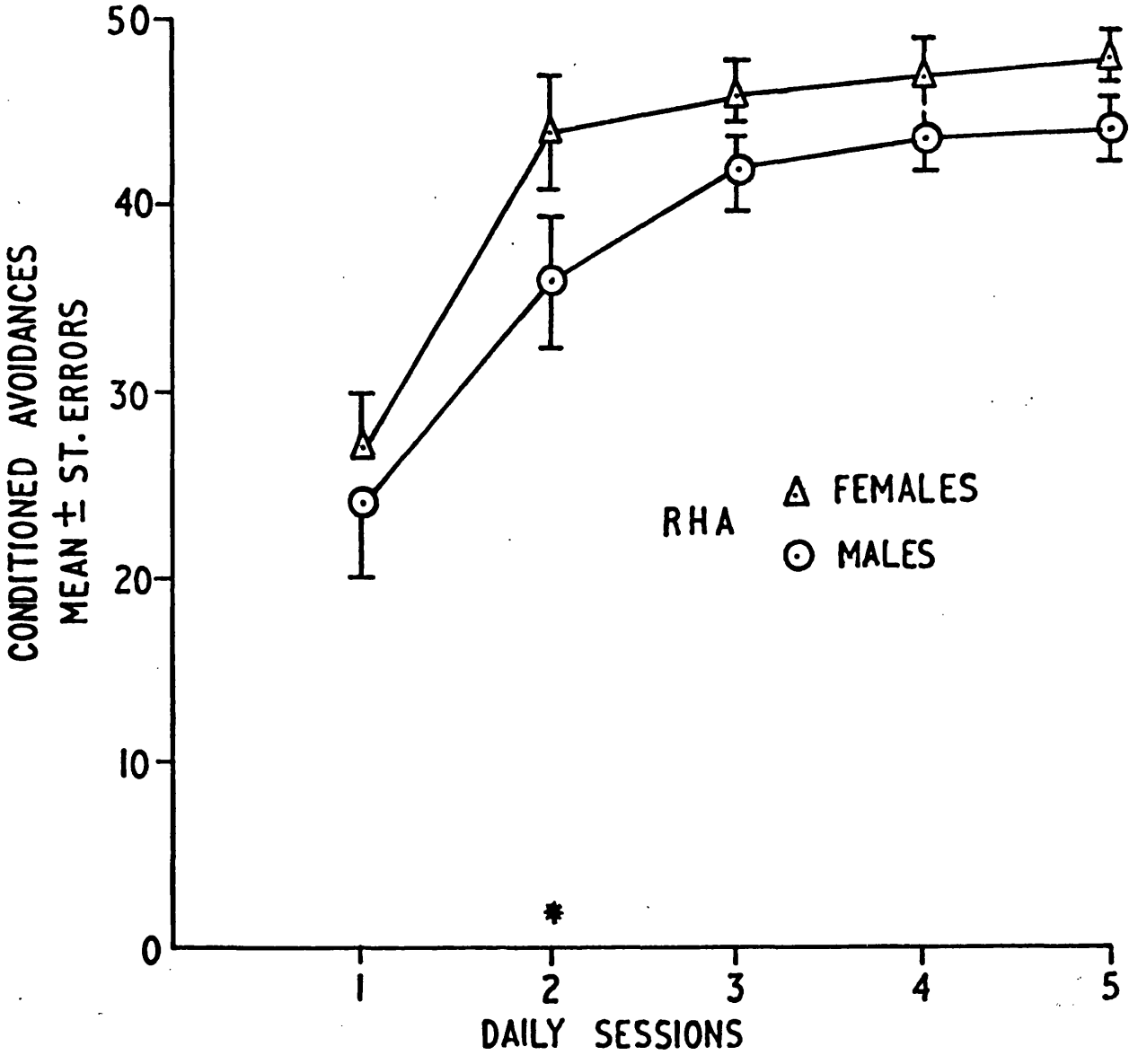


Figure 3-5. Avoidance learning in males and females of the RHA strain.

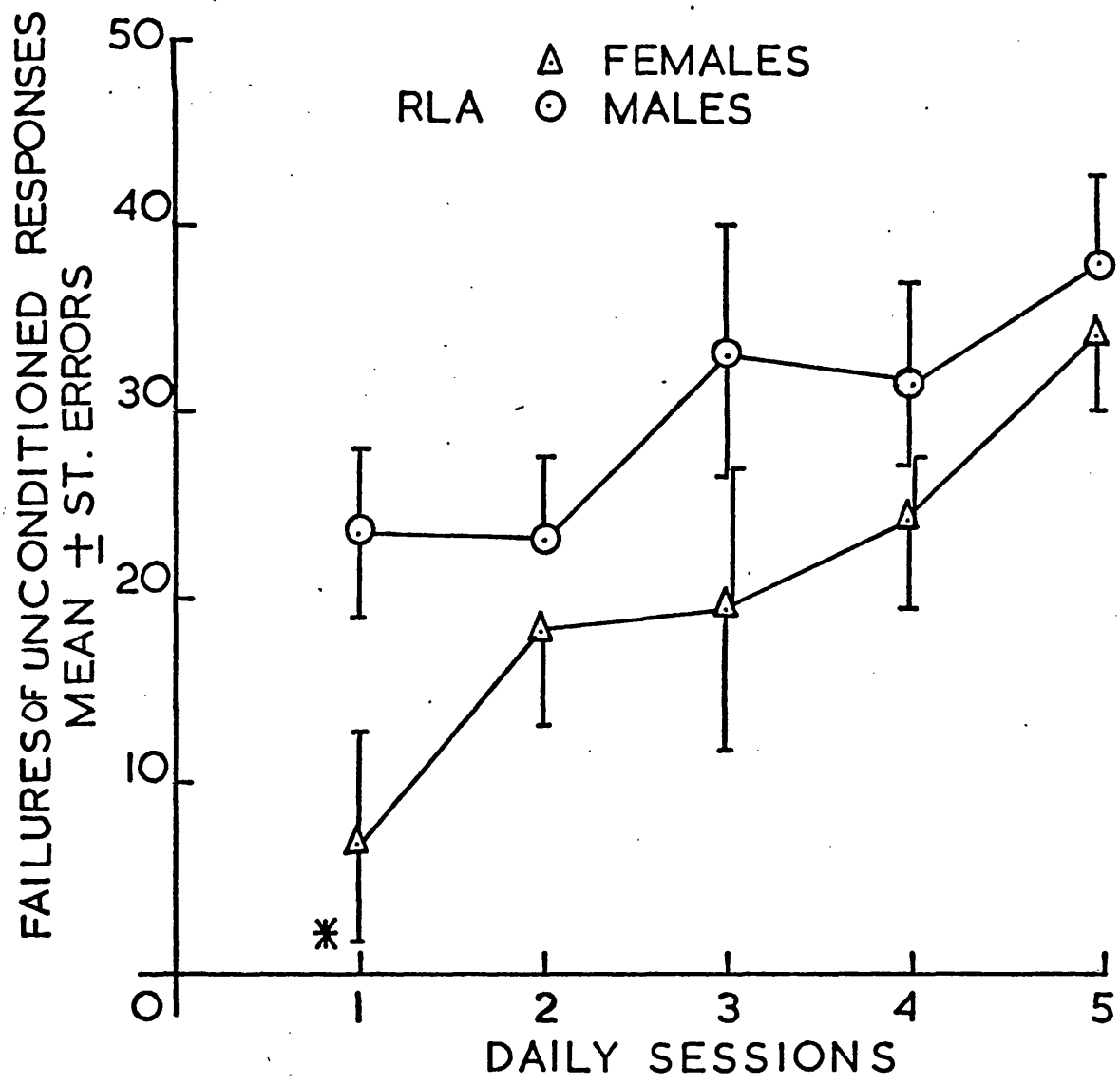


Figure 3-6. Avoidance learning in males and females of the RLA strain. Avoidance learning in the undrugged state in this strain was always close to zero. Means and standard errors of failures to perform unconditioned responses are shown.

3.3 Conditioned Avoidance Behaviour and Anti-ChE Drugs

Procedure

The effects of two anti-ChE agents, Physostigmine salicylate and Pyridostigmine hydrobromide, were examined on the acquisition of conditioned avoidance responding in rats of the 3 strains. Groups of 12 male rats from each of the strains were injected, subcutaneously (s.c.), with Physostigmine in doses of 0.125, 0.06 or 0.03 mg/kg., Pyridostigmine at a dose of 0.125 mg/kg. Drugs were dissolved in physiological saline and injected at 1.0 ml/kg. Control (saline) rats were trained in alternating sequency thorugh the day with the drug-treated rats. All rats were given five training sessions, spaced at 24 hour intervals and drug or saline was injected exactly 15 minutes before the beginning of each of sessions 1 - 4. (As all animals were allowed 5 minutes in the shuttlebox before training was started, the time between injection and training, was actually, 20 minutes). Session 5 was performed in the absence of both drug and saline injections. Thus the first 4 sessions examined learning over an extended period under the effects of the drug, and the final session examined the dependence of this behaviour on the presence of drug.

The results of the experiments with physostigmine are shown in Figures 3-7 to 3-15.

All the effects of physostigmine were manifested as depressions of conditioned avoidance behaviour. These effects were dose dependent and there was variation in the response of the strains to the drug at some of the doses used. The high dose (0.125 mg/kg.) produced severe depression of avoidance in Porton (Figure 3-7) and RHA (Figure 3-8) strain rats and reduction in escape responding of RLA rats (Figure 3-9). The figures for Porton and RHA rats also show the block of escape behaviour produced at this dose (right hand graph in each case). Note that Porton rats show an almost complete block of avoidance learning

and a very high incidence of block of escape responding (i.e., failures to make unconditioned responses). This implied a block of conditioned and unconditioned behaviour in these strains that is probably the result on a non-specific depression of behaviour. The RHA strain, however, show some learning during session 1 - 4 and both strains show an immediate return towards normal acquisition when the drug is withheld on the fifth day.

At the next largest dose used (0.06 mg/kg.) the depression of conditioning was less severe but strain differences in response were seen. The Porton strain (Figure 3-10) showed a depression of avoidance behaviour which was not significant, but also a decrease in escape behaviour which was significant in one of the sessions. The RHA rats (Figure 3-11) showed severely depressed conditioned avoidance behaviour which was significantly reduced below control level in all of the training sessions. The escape behaviour of this strain, however, was not significantly reduced at this dose.

RLA rats (Figure 3-12), at this dose, showed decreased escape behaviour which was significantly decreased in two of the sessions.

At the low dose (0.03 mg/kg.) neither Porton (Figure 3-13) nor RHA (Figure 3-14) strain animals showed any change in avoidance behaviour. RLA strain rats at this dose (Figure 3-15), however, showed significant reductions in escape behaviour.

The results of the experiments in which pyridostigmine was given before shuttlebox training are shown in Figure 3-16, Porton strain, Figure 3-17, RHA strain and Figure 3-18, RLA strain. No significant changes in avoidance conditioning were seen at this dose in any of the three strains.

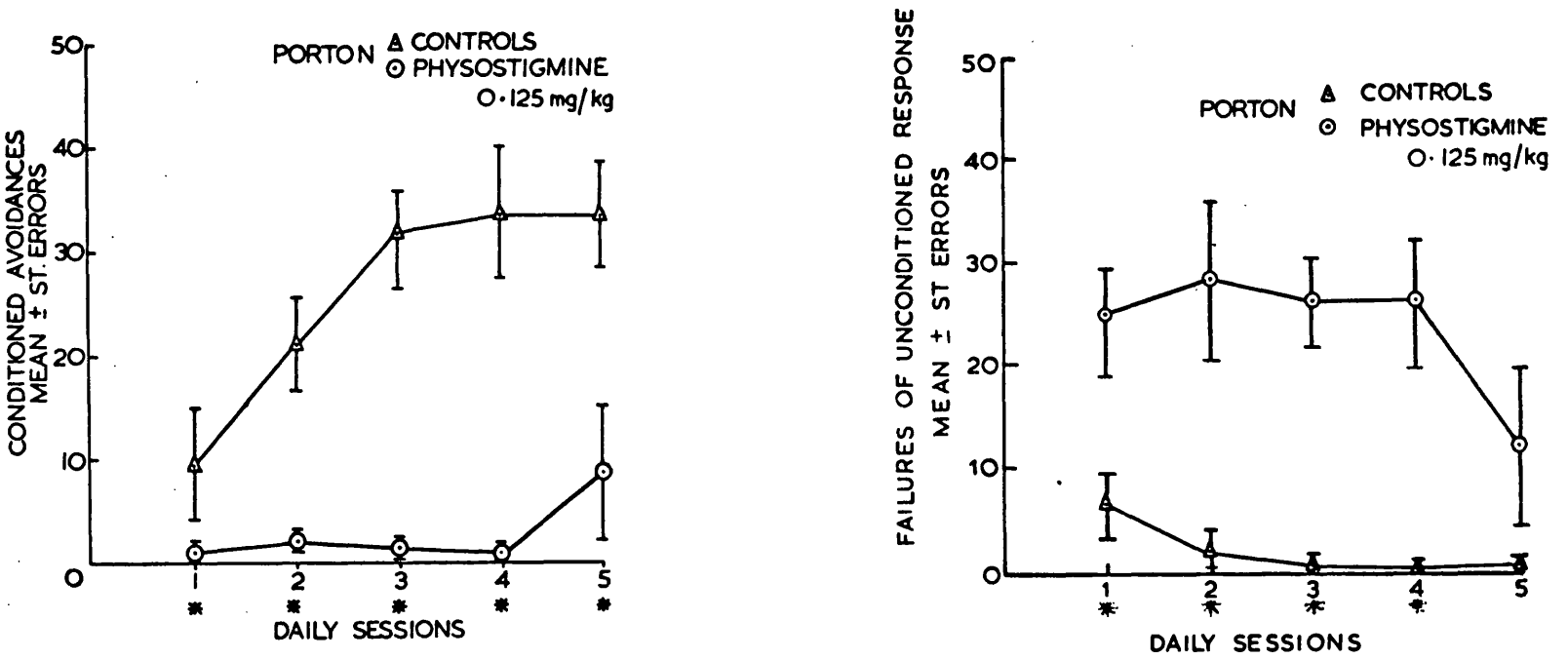


Figure 3-7. Conditioned avoidance learning in the Porton strain rat (10 males) after injection of Physostigmine (0.125 mg/kg.). Drug injected (s.c.), 15 minutes before beginning of sessions 1 - 4. No drug given before session 5. Conditioned avoidances (left-hand graph) and failures to make unconditioned responses (right-hand graph).

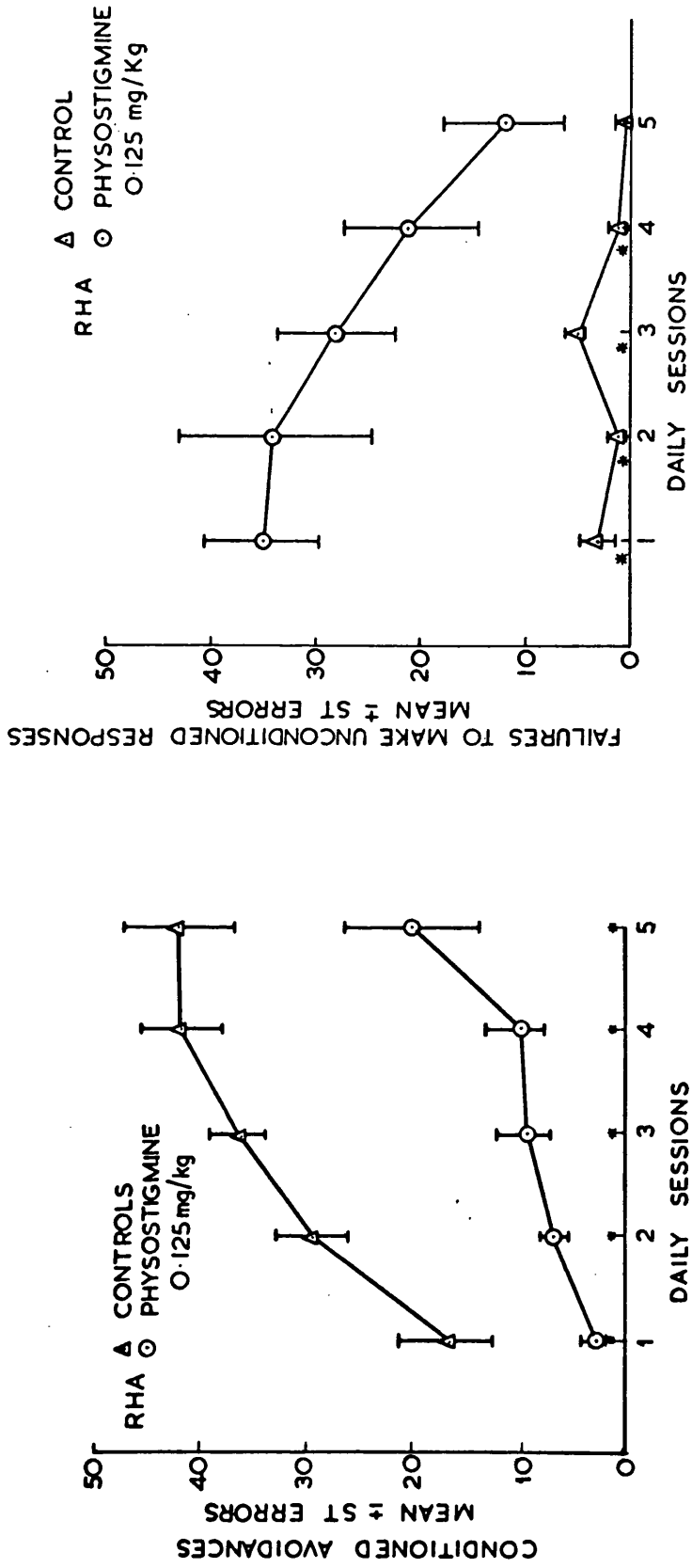


Figure 3-8. Conditioned avoidance learning in the RHA strain rat (10 males) after injection of Physostigmine (0.125 mg/kg.). Drug injected (s.c.), 15 minutes before beginning of sessions 1 - 4. No drug given before session 5. Conditioned avoidance (left-hand graph) and failures to make unconditioned responses (right-hand graph).

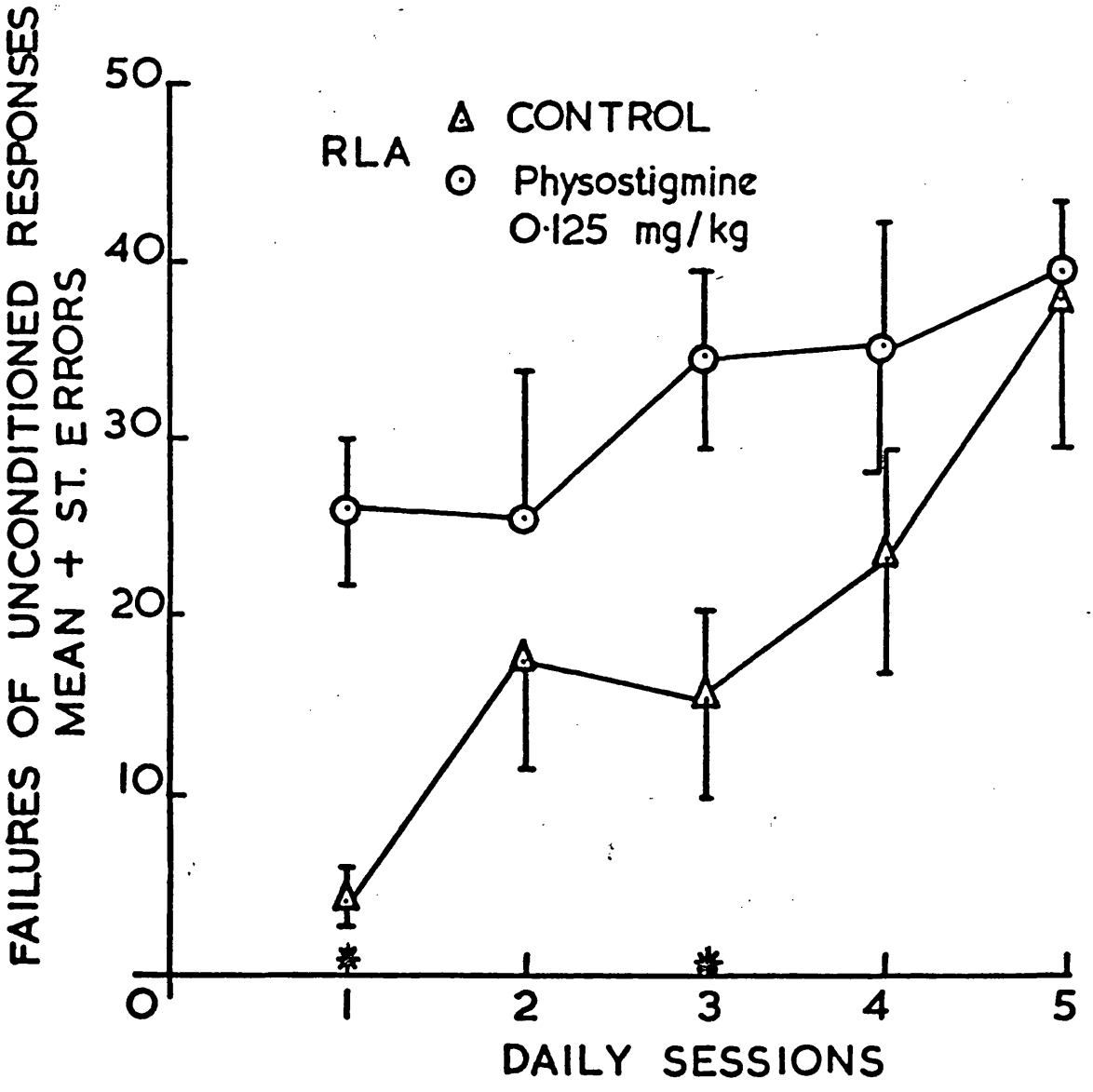


Figure 3-9. Avoidance learning in RLA strain males, injected (s.c.) with Physostigmine (0.125 mg/kg.), 15 minutes before each of sessions 1 - 4. No drug given before session 5. Note that points plotted are failures to respond to unconditioned stimulus.

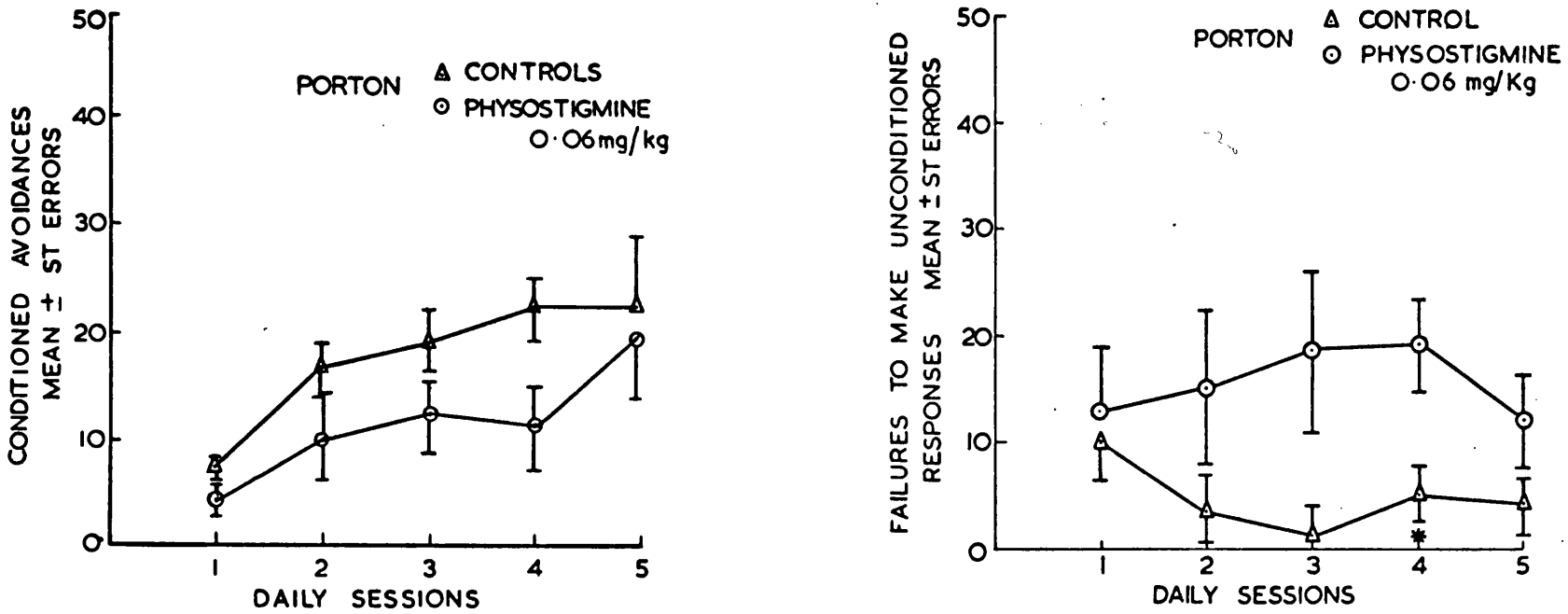


Figure 3-10. Conditioned avoidance learning in the Porton strain rat (10 males) after injection of Physostigmine (0.06 mg/kg.). Drug injected (s.c.), 15 minutes before beginning of sessions1 - 4. No drug given before session 5. Conditioned avoidances (left-hand graph) and failures to make unconditioned responses (right-hand graph).

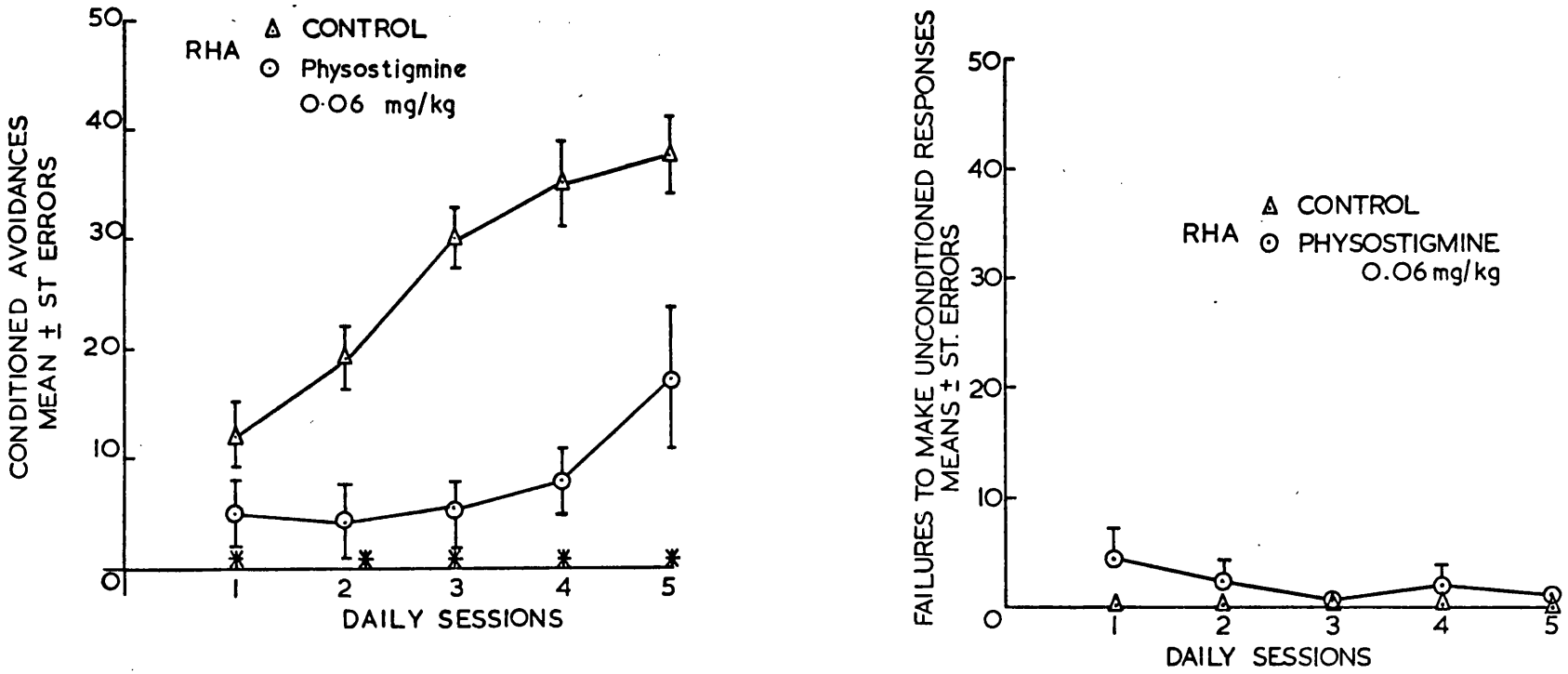


Figure 3-11. Conditioned avoidance learning in the RHA strain rat (10 males) after injection of Physostigmine (0.06 mg/kg.). Drug injected (s.c.), 15 minutes before beginning of sessions 1 - 4. No drug given before session 5. Conditioned avoidances (left-hand graph) and failures to make unconditioned responses (right-hand graph).

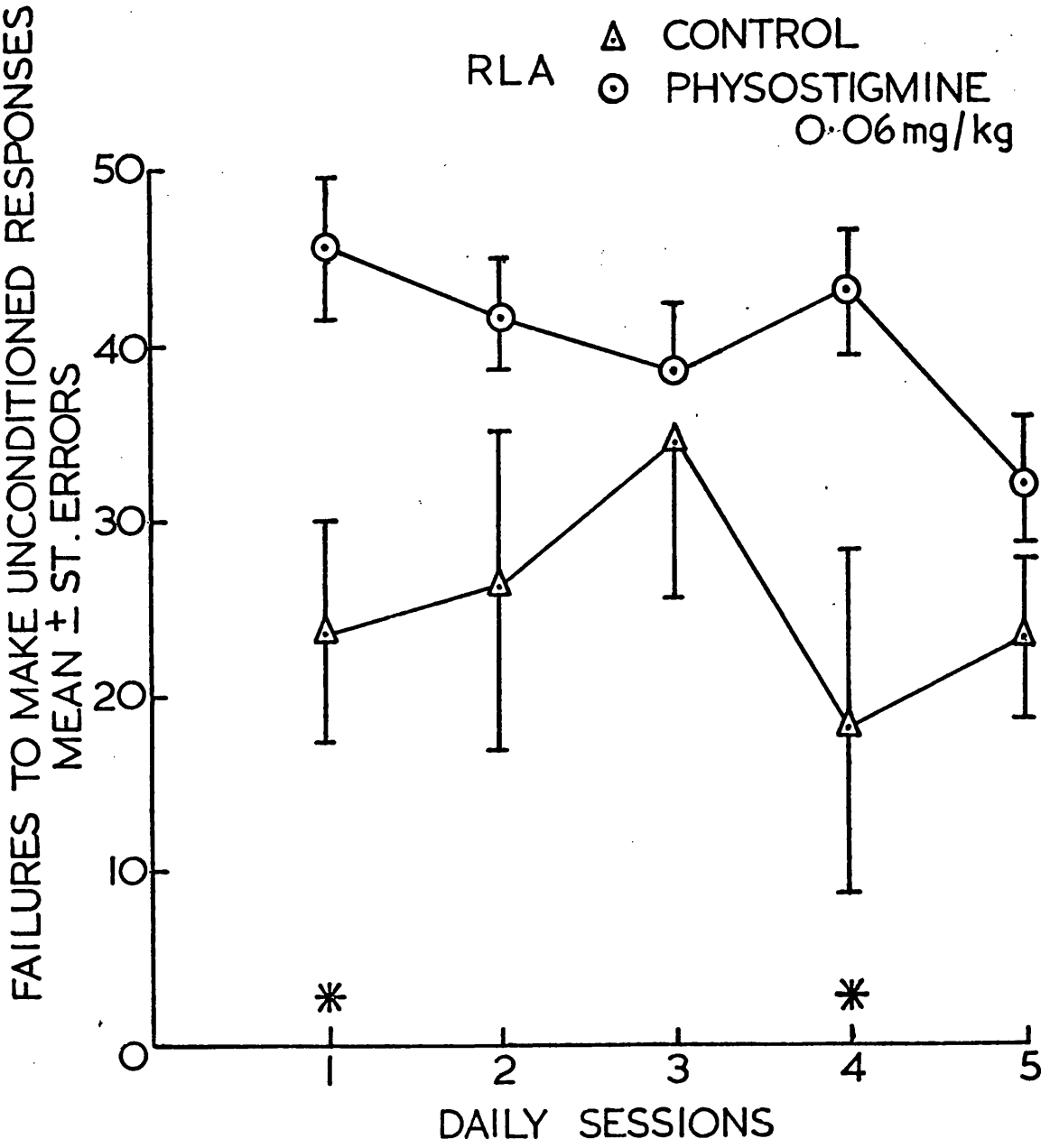


Figure 3-12. Conditioned avoidance learning in the RLA strain rat (10 males) after injection of physostigmine (0.06 mg/kg.). Drug injected (s.c.) 15 minutes before each of sessions 1 - 4. No drug given before session 5. Note that points plotted are failures to make unconditioned responses. Avoidance responding was not significantly different from zero.

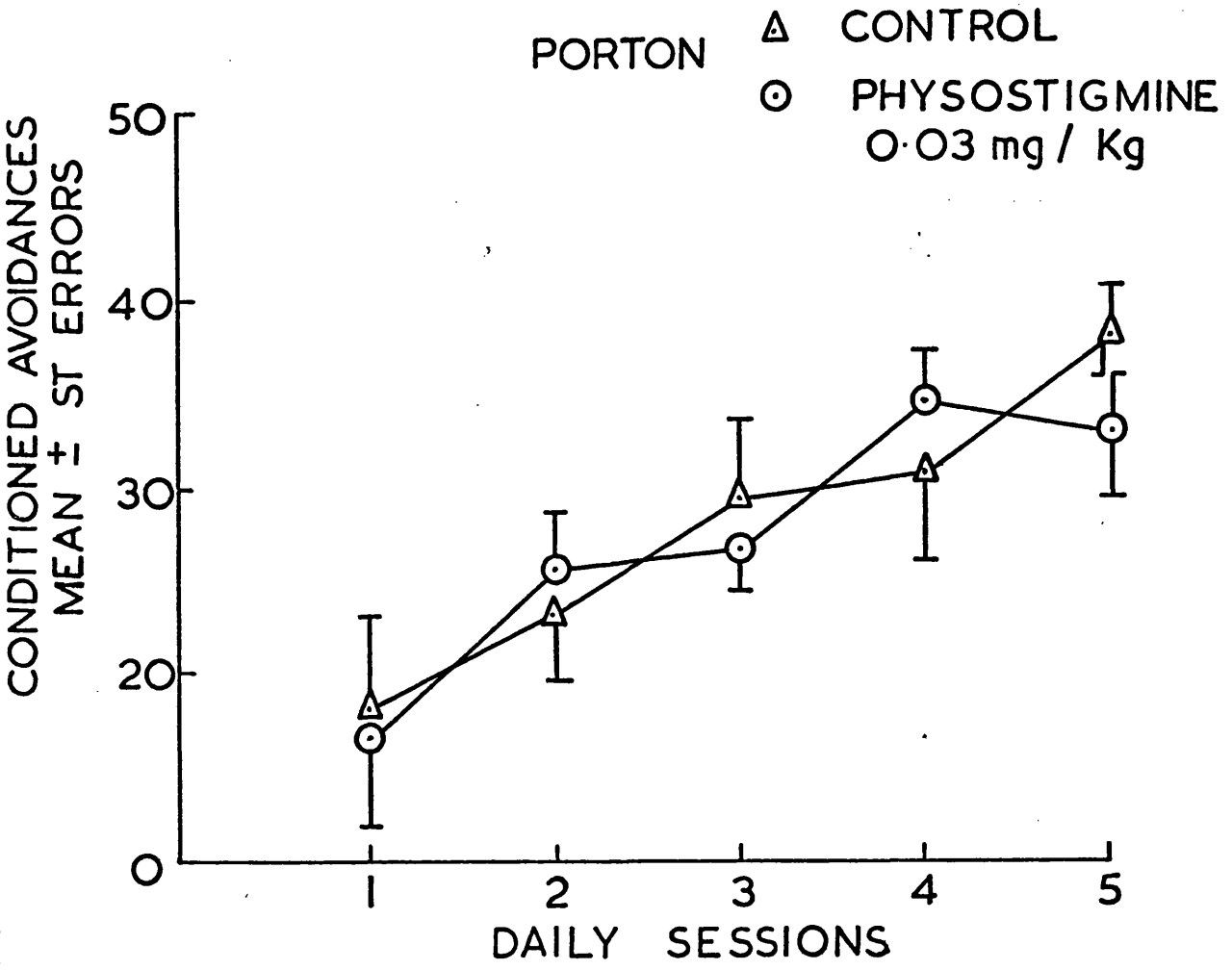


Figure 3-13. Conditioned avoidance learning in the Porton strain rat (10 males) after injection of Physostigmine (0.03 mg/kg.). Drug injected (s.c.), 15 minutes before beginning of sessions 1 - 4. No drug given before session 5.

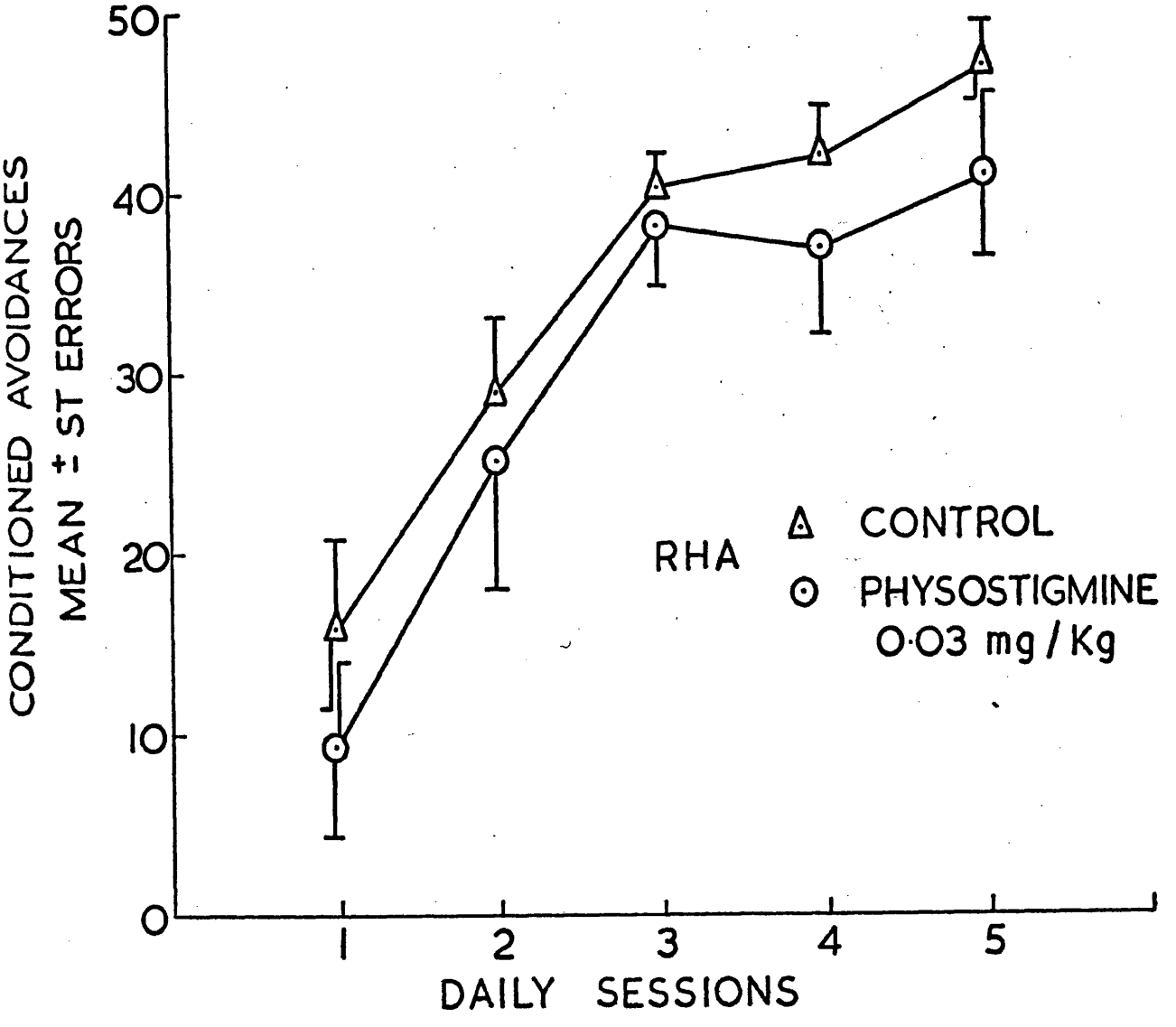


Figure 3-14. Conditioned avoidance learning in the RHA strain rat (10 males) after injection of Physostigmine (0.03 mg/kg.). Drug injected (s.c.), 15 minutes before beginning of sessions 1 - 4. No drug given before session 5.

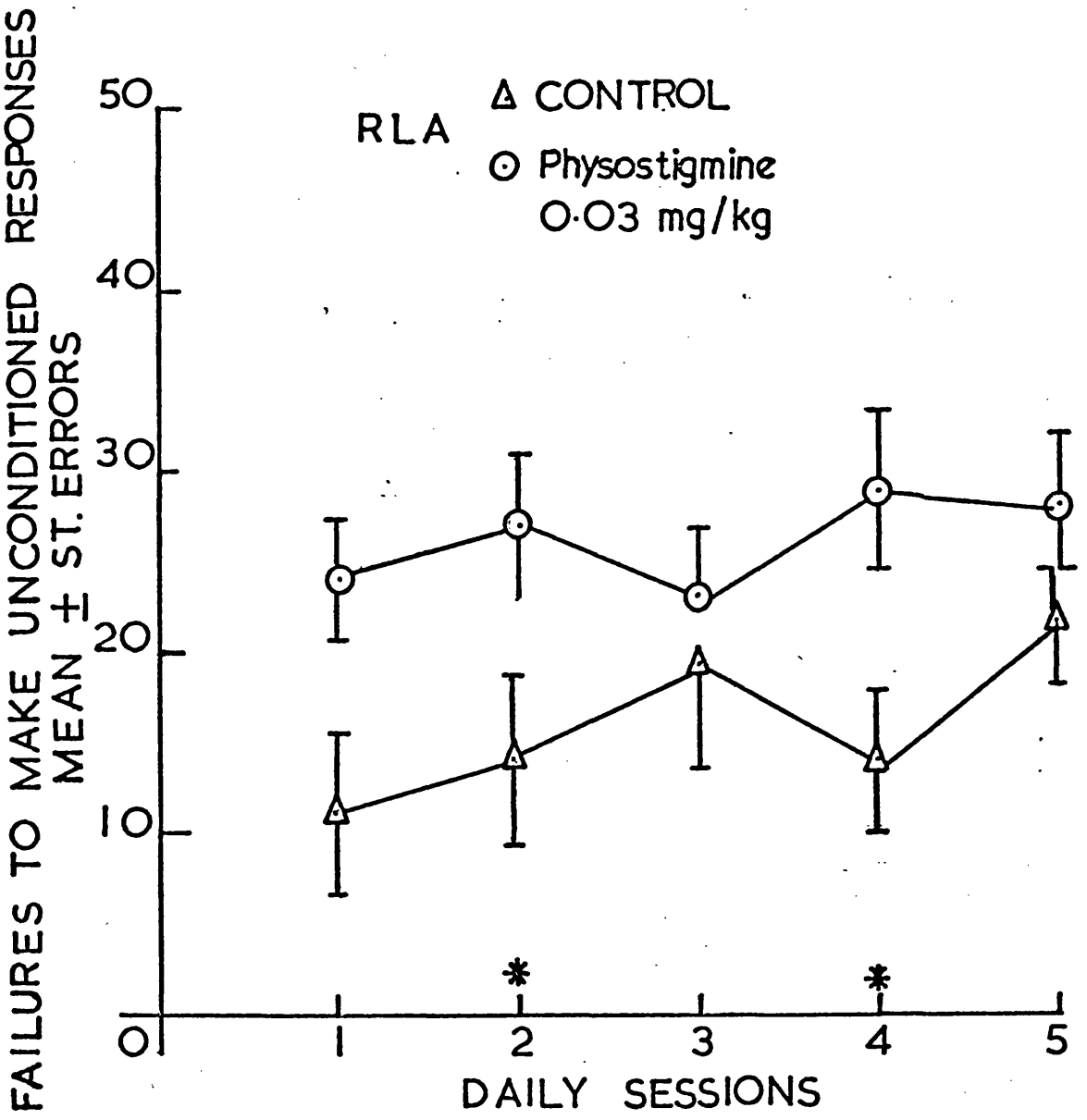


Figure 3-15. Conditioned avoidance learning in the RLA strain rat (10 males) after injection with Physostigmine (0.03 mg/kg.). Drugs injected (s.c.), 15 minutes before sessions 1 - 4. No drug given before session 5. Note that points plotted are failures to make unconditioned responses. Avoidance responding was not significantly different from zero.

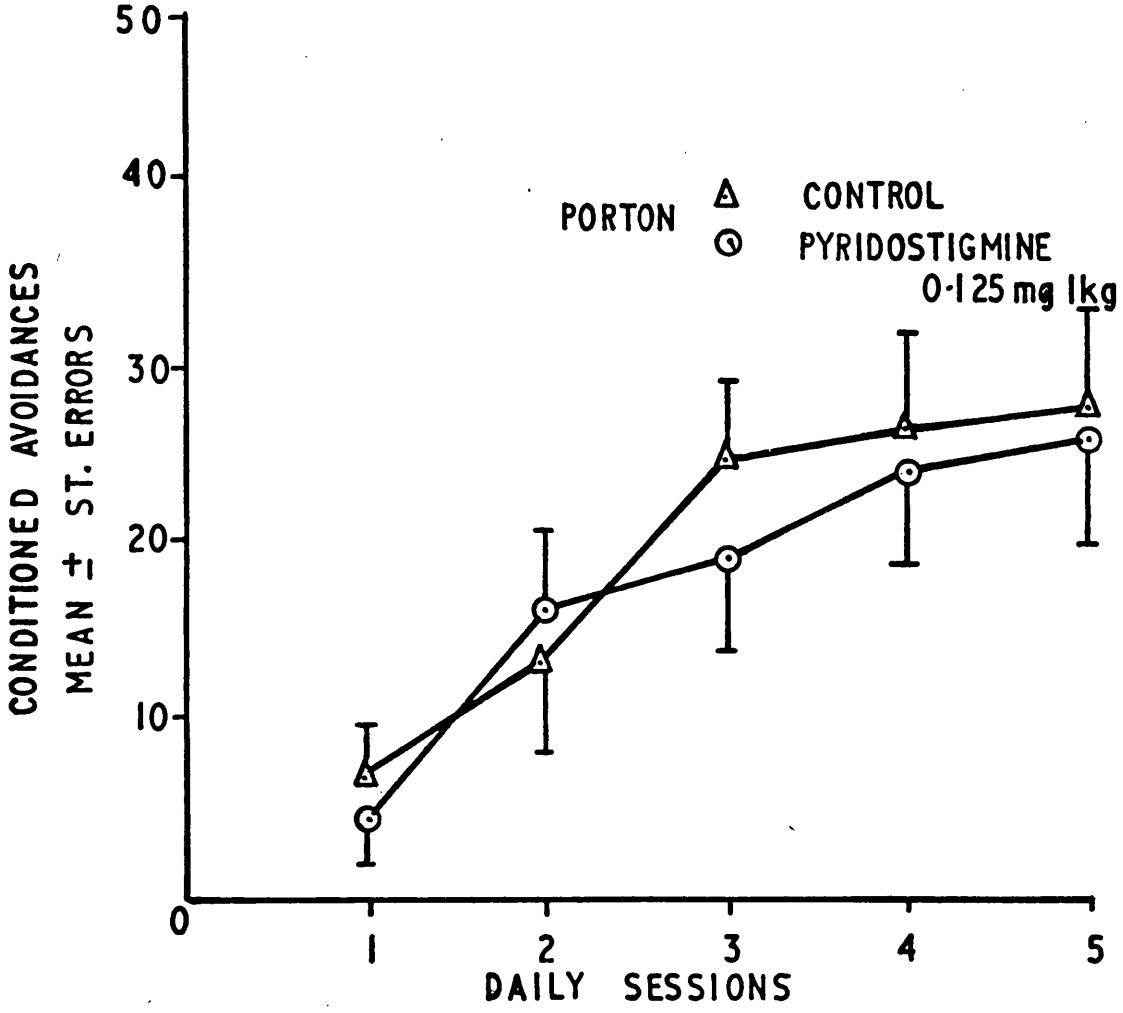


Figure 3-16. Avoidance learning in Porton strain males, after injection (s.c.) with Pyridostigmine (0.125 mg/kg.), 15 minutes before sessions 1 - 4. No drug given before session 5.

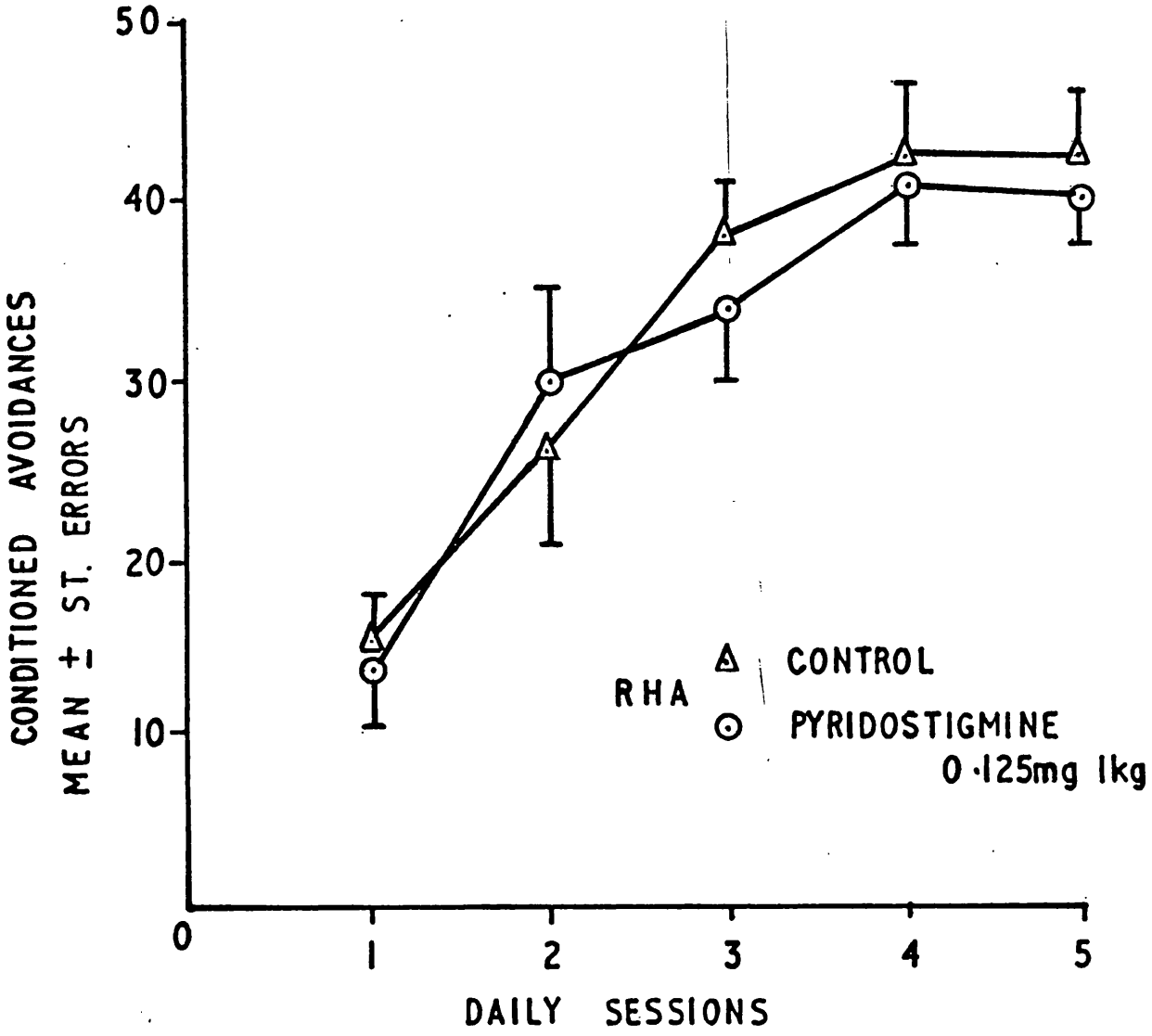


Figure 3-17. Avoidance learning in RHA strain males after injection (s.c.) with Pyridostigmine (0.125 mg/kg.), 15 minutes before sessions 1 - 4. No drug given before session 5.

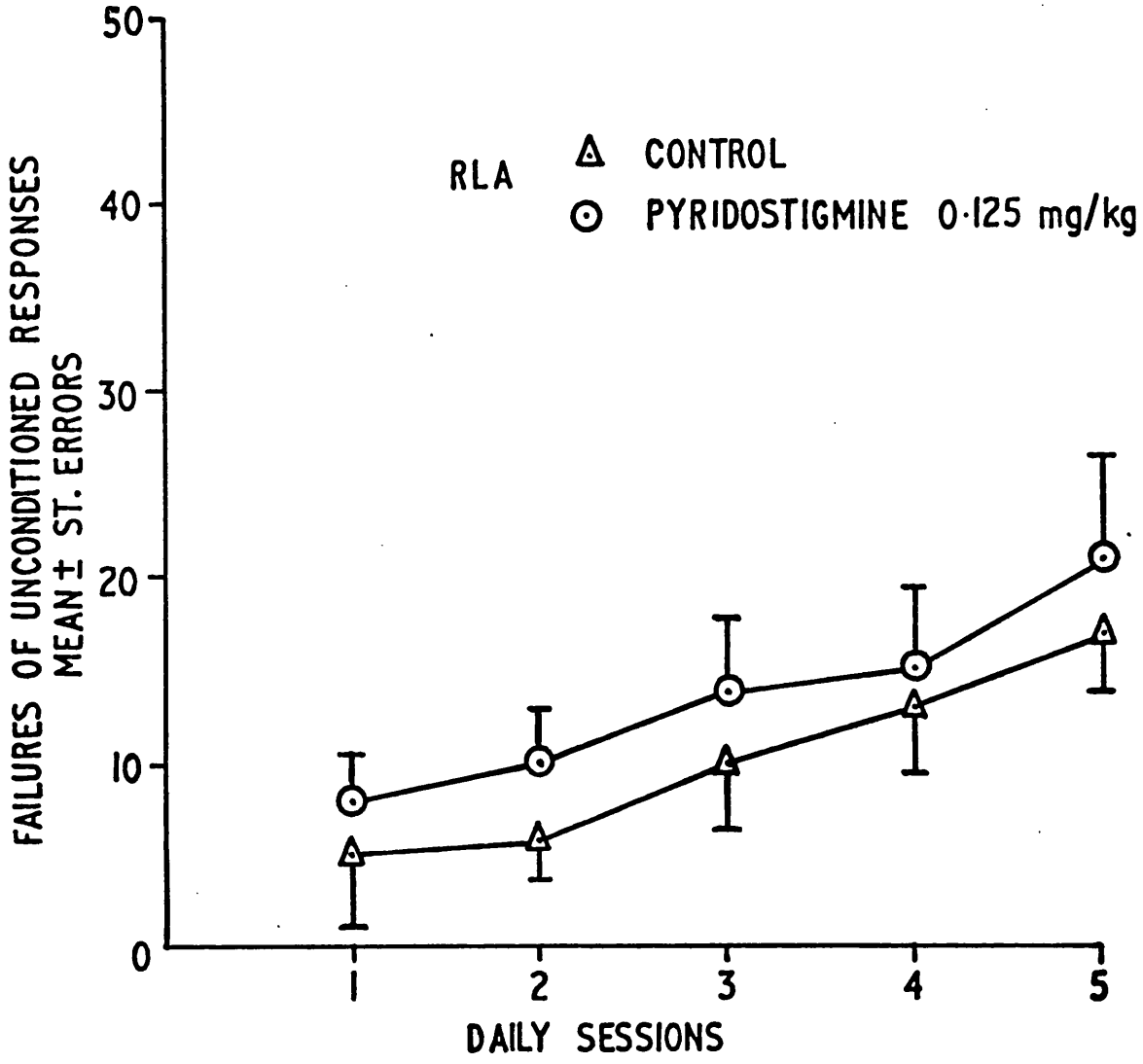


Figure 3-18. Avoidance learning of RLA strain males injected (s.c.) with Pyridostigmine (0.125 mg/kg.), 15 minutes before each of sessions 1 - 4. No drug given before session 5. Note that points plotted represent failures to respond to unconditioned stimulus. Conditioned avoidances are equal to zero.

3.4 Conditioned Avoidance Behaviour and Anti-ACh Drugs

3.41 Shuttlebox Experiments

Procedure

The effects of two anti-ACh drugs, NEPB and NEPB MeI were examined on the acquisition of conditioned avoidance behaviour in rats of the three strains. Procedure was similar to that described for anti-ChE drugs and the doses were as follows, NEPB 1.0 mg/kg. (i.p.) and NEPB MeI, 1.0 mg/kg. (i.p.), injected 15 minutes before placing in the shuttlebox on each of sessions 1 - 4.

The results of the experiments with NEPB are shown in Figure 3-19, Porton strain, Figure 3-20, RHA strain and Figure 3-21, RLA strain.

The effect seen in all strains was facilitation of conditioning. Significantly increased avoidance responding was seen after NEPB in Porton and RHA strains. The high level of responding was maintained in RHA but not in the Porton strain rats on the fifth day when drug was no given. The RLA strain showed significantly improved conditioned behaviour. Failures in escape behaviour were reduced and a low level of avoidance responding was seen which was significantly different from control (although normal avoidance responding in this strain is nearly always zero). Both types of response reverted to control level when drug was withheld on the fifth day.

The effects on avoidance conditioning of NEPB MeI in the strains is shown in Figure 3-22, Porton strain, Figure 3-23, RHA strain and Figure 3-24, RLA strain. Reduced avoidance responding was seen in Porton and RHA strain rats although this only reached significant levels in the RHA strain. The RLA rats also showed a reduction in responding, seen as a decrease in escape responding. This effect was significant in one of the sessions.

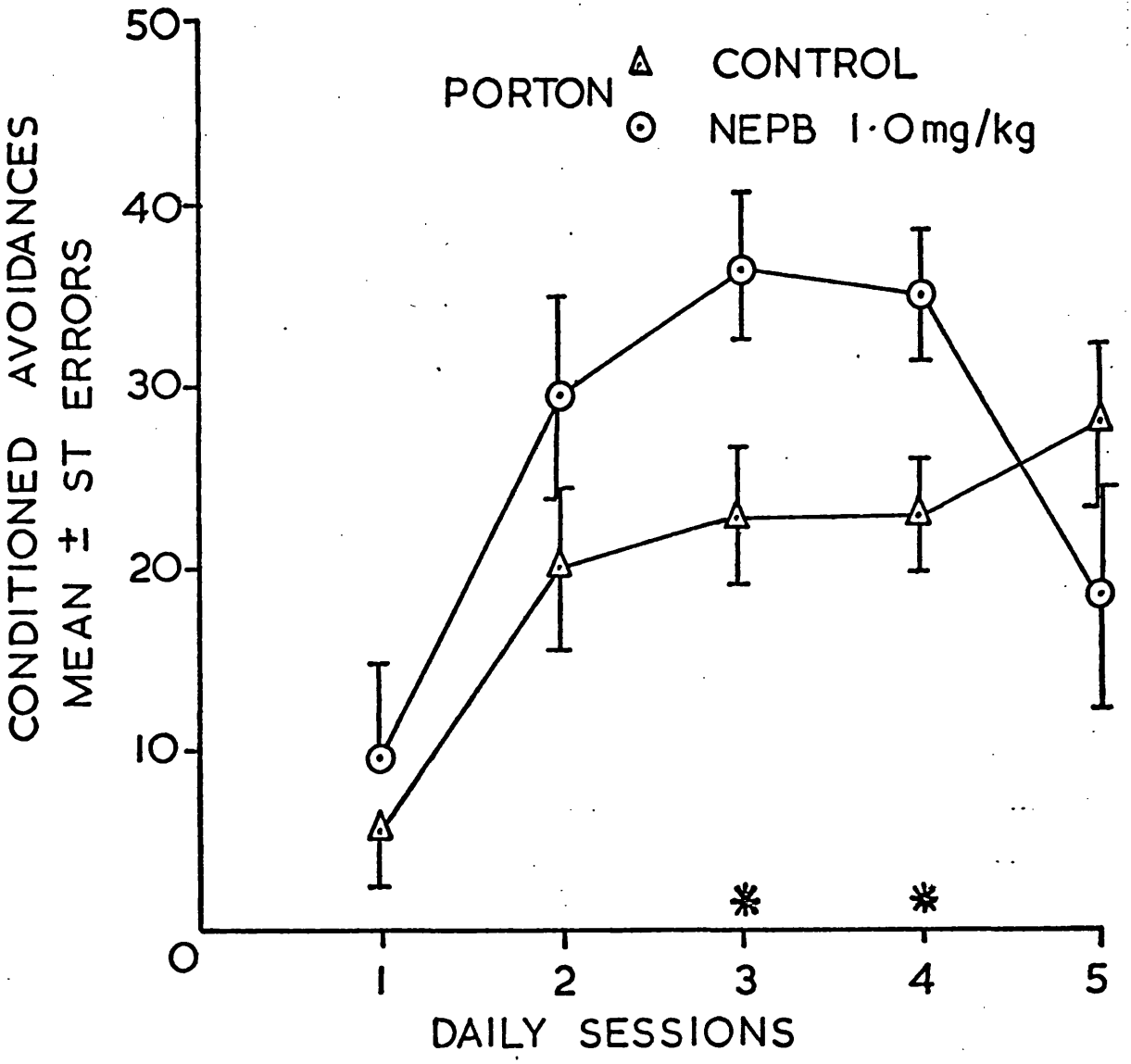


Figure 3-19. Conditioned avoidance learning in Porton strain rats (8 males) after injection of NEPB (1.0 mg/kg.). Drug was injected (i.p.) 15 minutes before sessions 1 - 4. No drug was given before session 5.

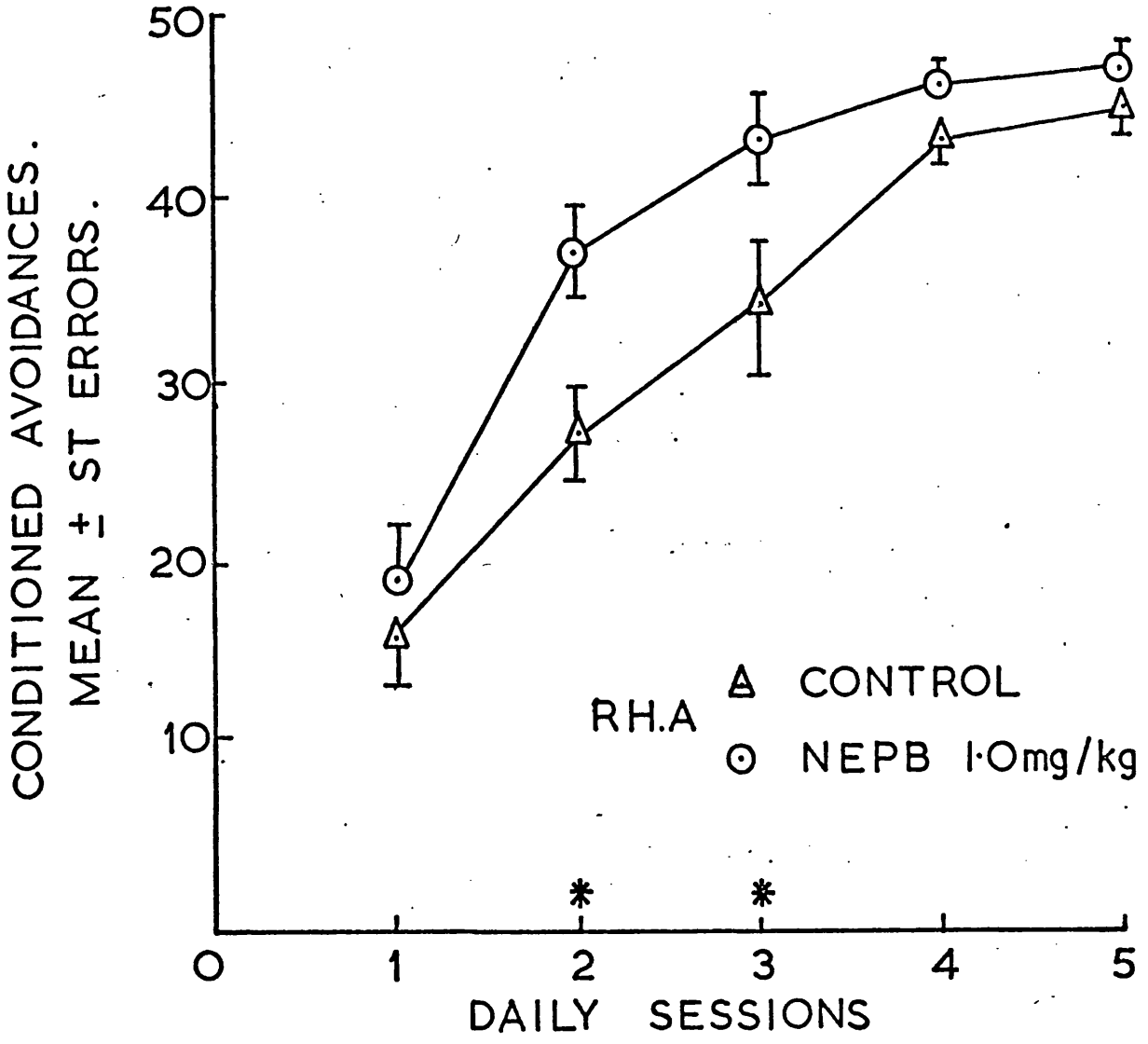


Figure 3-20. Conditioned avoidance learning in RLA strain rats (10 males) after injection of NEPB (1.0 mg/kg.). Drug was injected (i.p.) 15 minutes before session 1 - 4. No drug was given before session 5.

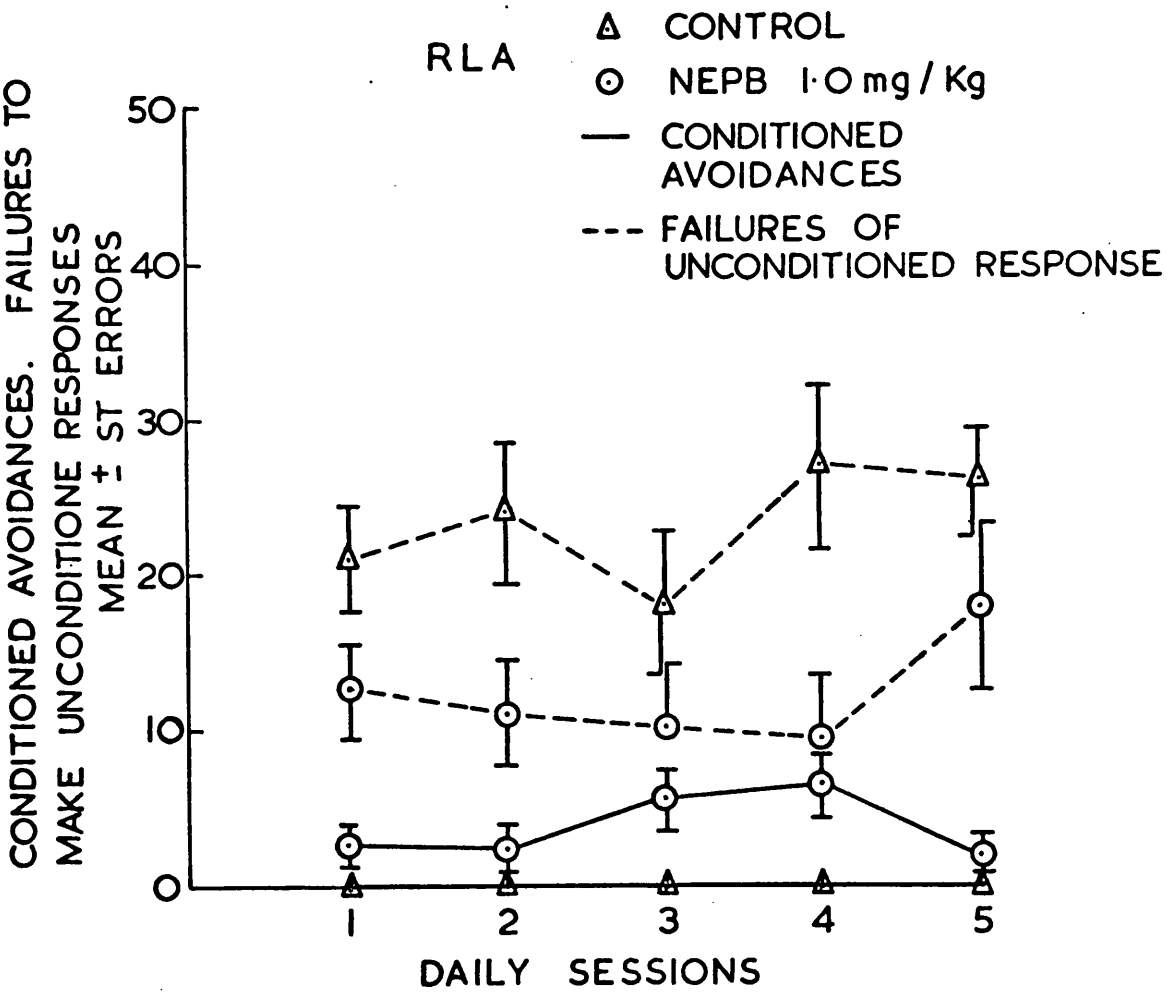


Figure 3-21. Conditioned avoidance learning in the RLA strain rat (10 males) after injection of NEPB (1.0 mg/kg.). Drug injected (i.p.) 15 minutes before each of session 1 - 4. No drug given before session 5. Note that points plotted are failures to make unconditioned responses and conditioned avoidances. Significantly different means are not shown as such on the figure, but are as follows: Conditioned avoidances significantly increased above control, in sessions 3 and 4; 'failures', significantly reduced below control in sessions 1 - 4.

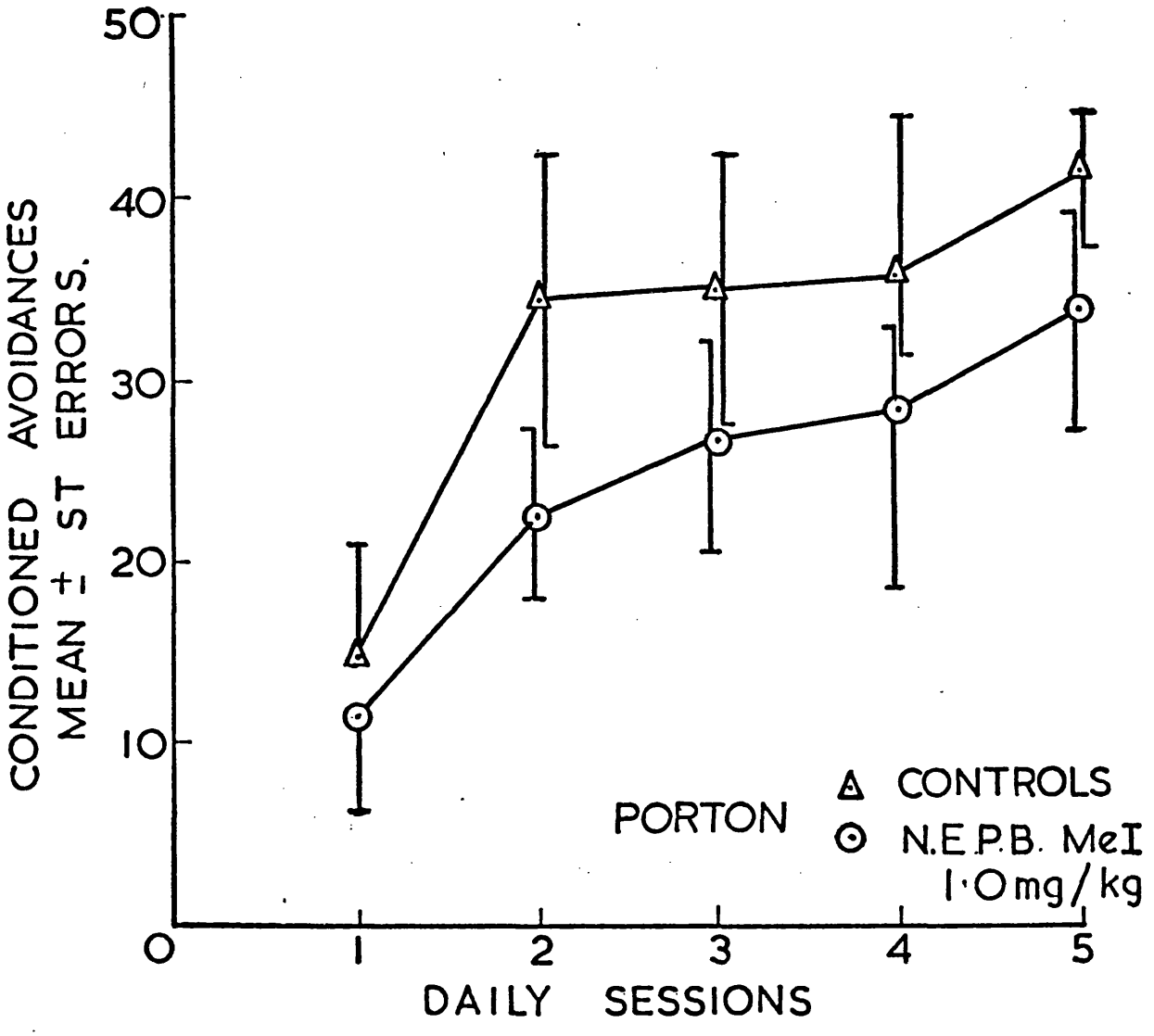


Figure 3-22. Avoidance learning in Porton strain males after injection with NEPB MeI (1.0 mg/kg.), 15 minutes before each of sessions 1 - 4. No drug given before session 5.

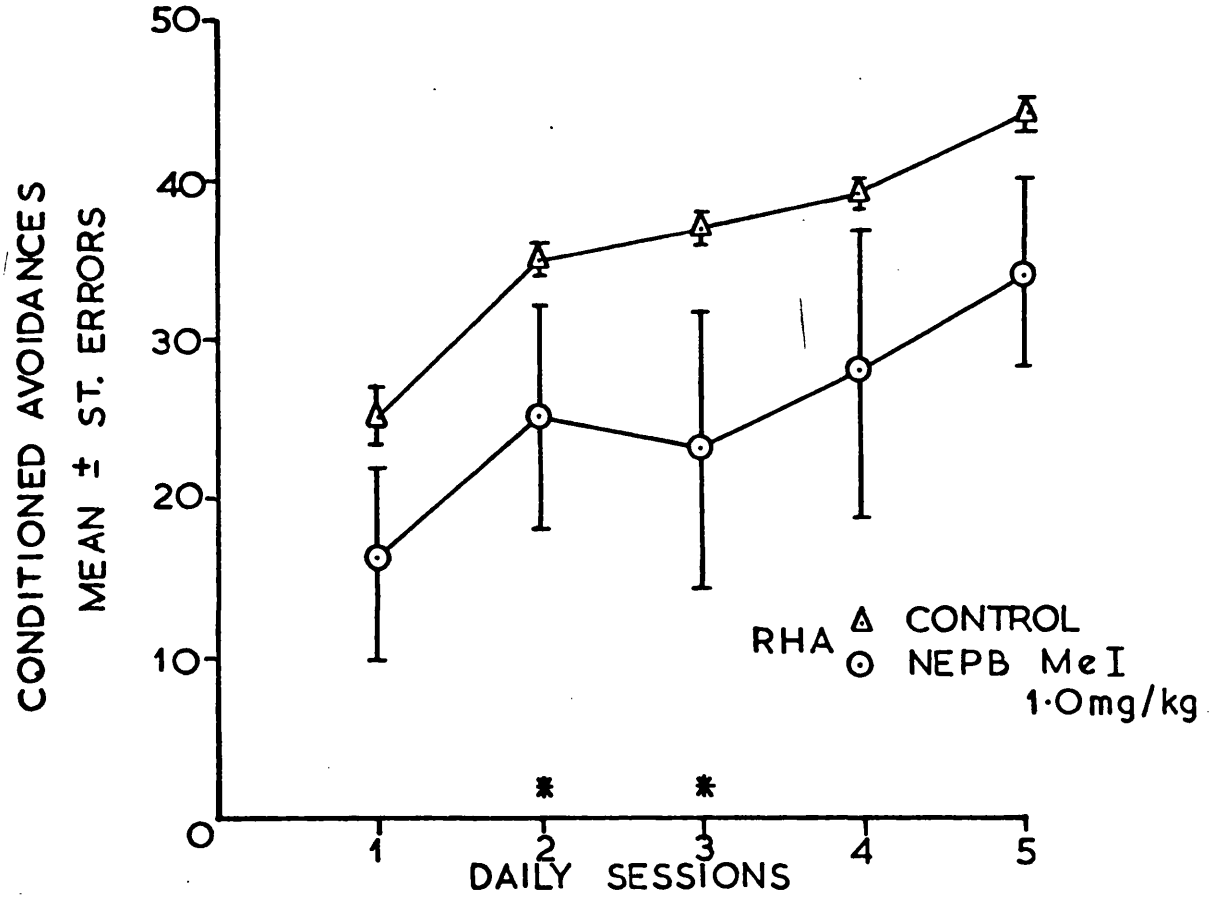


Figure 3-23. Conditioned avoidance learning in the RHA strain rat (10 males) after injection (i.p.) with NEPB MeI (1.0 mg/kg.). Drug injected 15 minutes before each of sessions 1 - 4. No drug given before session 5.

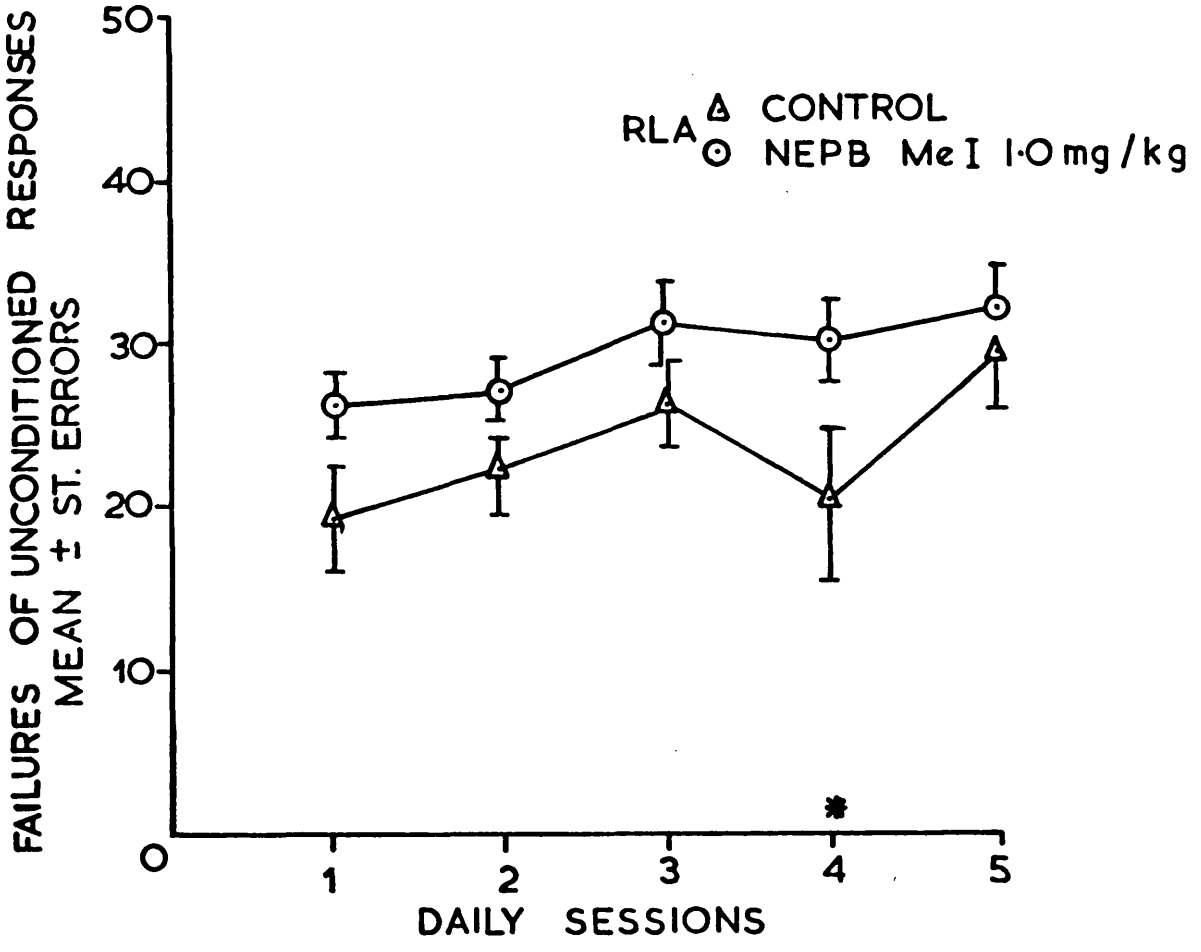


Figure 3-24. Avoidance learning in RLA strain males, injected (i.p.) with NEPB MeI (1.0 mg/kg.), 15 minutes before each of sessions 1 - 4. No drug given before session 5. Note that points plotted are failures to respond to unconditioned stimulus.

3.42 Pain-Threshold Experiment

Anti-ACh drugs are known to produce drying of the skin and since dry skin may have a higher electrical resistance than normal skin, the pain-threshold of rats given anti-ACh drugs may be higher than that of normal animals. A simple experiment was conducted to compare the pain-thresholds to shock of normal rats with those given anti-ACh drugs.

Procedure

Six rats from each of the three strains were placed, singly, into a compartment of the, slightly modified, shuttlebox. Access to the other compartment was prevented by a temporary barrier, taped across the connecting door and an alternative shock source was used which provided a facility for varying the level of shock applied to the grid. The shock level at which rats first showed signs of discomfort was then determined as follows; the shock unit was set to give a low level, shock stimulus and whilst watching the rat, the shock was turned on. The shock was applied long enough to produce a response (few seconds) and then turned off. If no response was produced the shock level was increased and applied again and so on, until responses were produced. Two shock levels were recorded for each animal, (i) the level required to produce a small 'flinch' response, and (ii) that required to promote active escape movements which were usually accompanied by vocalisation.

Pain-thresholds were determined in this way for each of the three strains and which were then divided into two groups, for drug treatment. Three rats of each strain were injected (i.p.), with NEPB (1.0 mg/kg.) and three with NEPB MeI (1.0 mg/kg.), 30 minutes before determining the pain-thresholds for a second time. 3 hours after drug treatment the pain-thresholds were determined for a third time. No significant differences in pain-threshold were found between the strains, in the normal

or drugged groups, so results were pooled to give statistically more meaningful group sizes. The results are shown below in which the means and standard deviations shown, represent arbitrary shock-unit, dial settings which represent, approximately, volts x 10.

	Pain-thresholds			
	Control (1)	NEPB	NEPB MeI	Control (2)
Shock level (mean ± standard deviation)	23.9 ± 1.4	20.6 ± 1.1	38.9 ±6.3**	25.5 ± 4.0

Rats treated with NEPB MeI, 30 minutes before the experiment showed a pain-threshold that was signficantly (Student's t-test $P < 0.01$) increased above that measured in the same rats before and after drug treatment and above that of rats given a similar dose of NEPB.

3.5 Conditioned Avoidance Behaviour and d-Amphetamine

Procedure

The effect of d-Amphetamine on conditioned avoidance behaviour was investigated in the three strains. The procedure was similar to that described for the previous shuttlebox experiments and d-Amphetamine was injected, s.c., at a dose of 0.1 mg/kg., 15 minutes before the beginning of session 1 - 4, but not before session 5. A control group received saline (1.0 mg/kg.), s.c., before training.

The results of these experiments are described in Figure 3-25, Porton strain, Figure 3-26, RHA strain and Figure 3-27, RLA strain. The effects seen in all strains was facilitation of avoidance conditioning. The effect in Porton and RHA animals consisted of significantly enhanced responding in the first 2 or 3 sessions, whilst RLA rats showed improved escape responding (failures of unconditioned behaviour reduced in all drug sessions) and low level (significant in 1 session), avoidance responding.

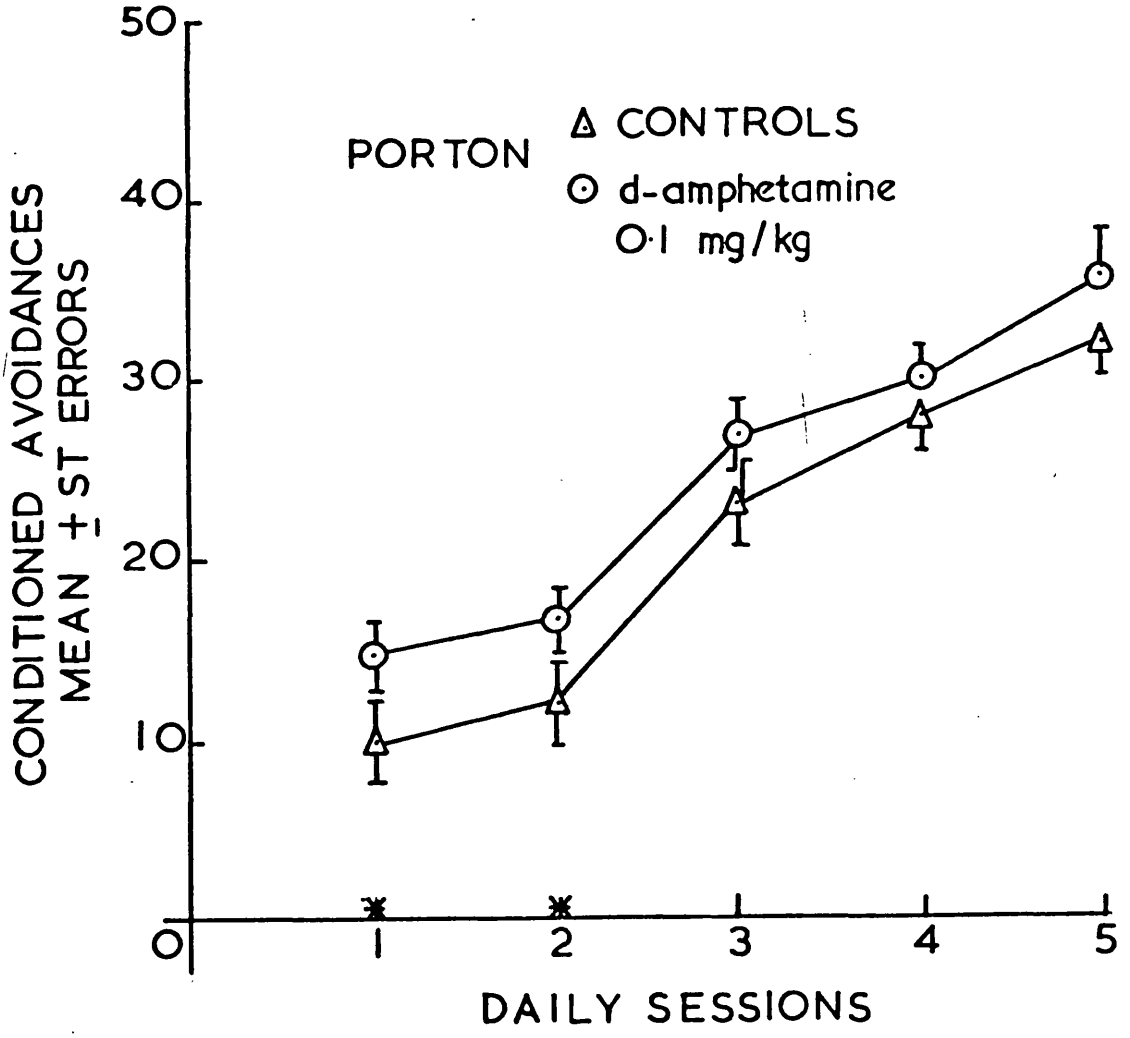


Figure 3-25. Conditioned avoidance learning in Porton strain rats after injection with d-Amphetamine (0.1 mg/kg.) Drug injected (s.c.) 15 minutes before beginning of sessions 1 - 4. No drug given before session 5.

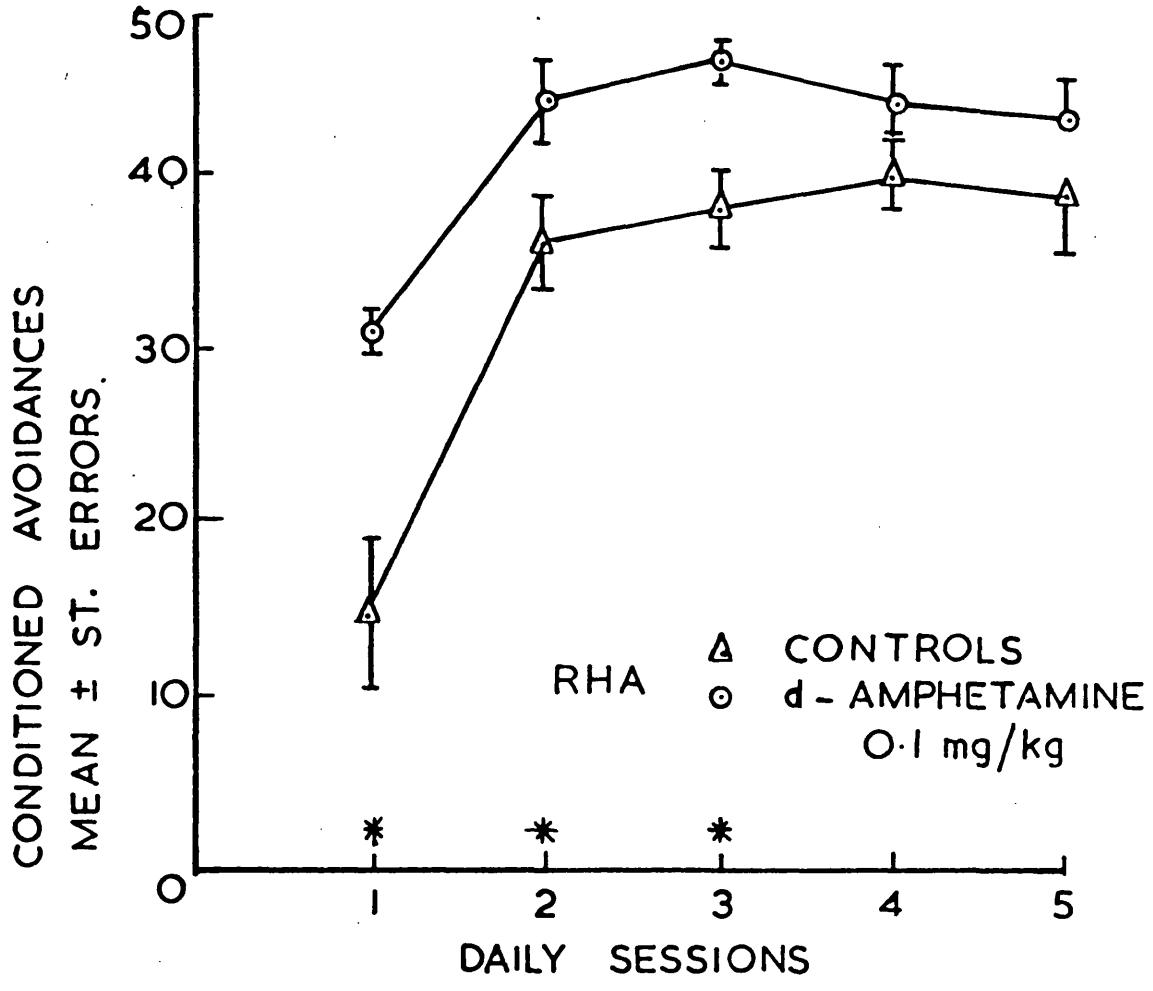


Figure 3-26. Avoidance learning in RHA strain males, after injection (s.c.) with d-Amphetamine (0.1 mg/kg.), 15 minutes before each of sessions 1 - 4. No drug given before session 5.

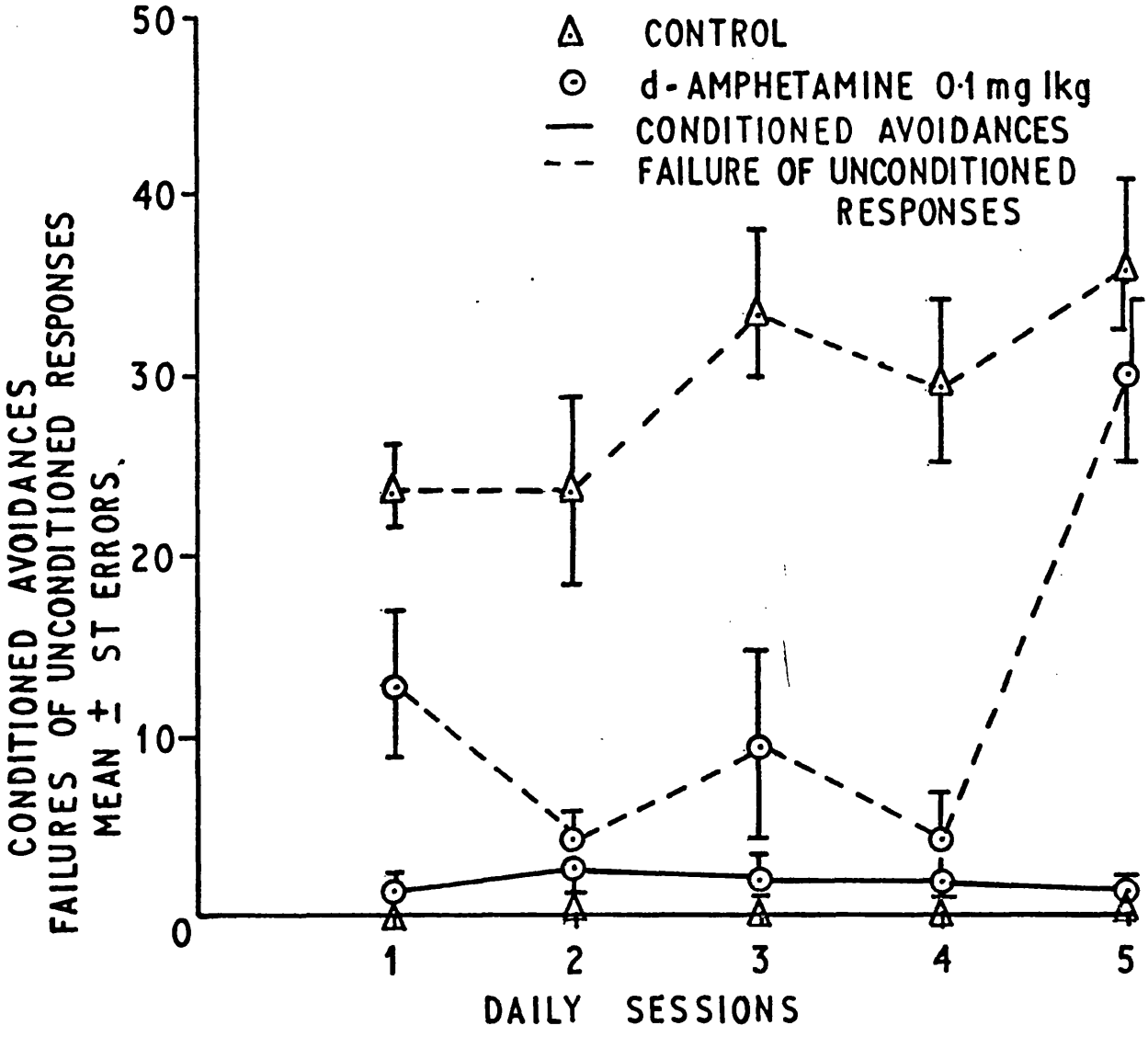


Figure 3-27. Avoidance learning of RLA strain males injected (s.c.) with d-amphetamine (0.1 mg/kg.), 15 minutes before each of sessions 1 - 4. No drug given before session 5. Conditioned avoidances and failures to respond to unconditioned stimulus are plotted. Significant differences are not shown on the graph but are as follows: Failures of unconditioned responses, significant in sessions 1 - 4. Conditioned avoidances significant in session 2.

3.6 Conditioned Avoidance Behaviour after Combined Treatment with Anti-ACh and Adrenergic Drugs

Procedure

The effect on conditioned avoidance behaviour of giving a combination of NEPB and d-Amphetamine at doses, which given separately produce no significant changes in conditioned avoidance behaviour, was investigated in the three strains. It was discovered in pilot experiments, that NEPB at a dose of 0.75 mg/kg., and d-Amphetamine at a dose of 0.075 mg/kg., did not significantly change conditioned avoidance behaviour in the strains and were therefore, chosen for use in this study.

NEPB (0.75 mg/kg.) was injected, i.p., followed immediately by d-Amphetamine (0.075 mg/kg.) s.c., 15 minutes before the beginning of sessions 1 - 4. No drugs were given before session 5.

The results of these experiments are shown in Figure 3-28, Porton strain, Figure 3-29, RHA strain and Figure 3-30, RLA strain. The overall effect was one of enhanced responding with varying grades of enhancement between the strains. The RHA rats showed slight enhancement of avoidance responding but the effect was not significant. The Porton rats, however, showed improved conditioning to a greater extent which was significantly different from control in 2 sessions.

The RLA rats again showed a level of avoidance responding after drug treatment which was significantly greater than control in several sessions. Avoidance responding returned to zero in session 5 when the drug was withheld. Escape responding was also facilitated under drug.

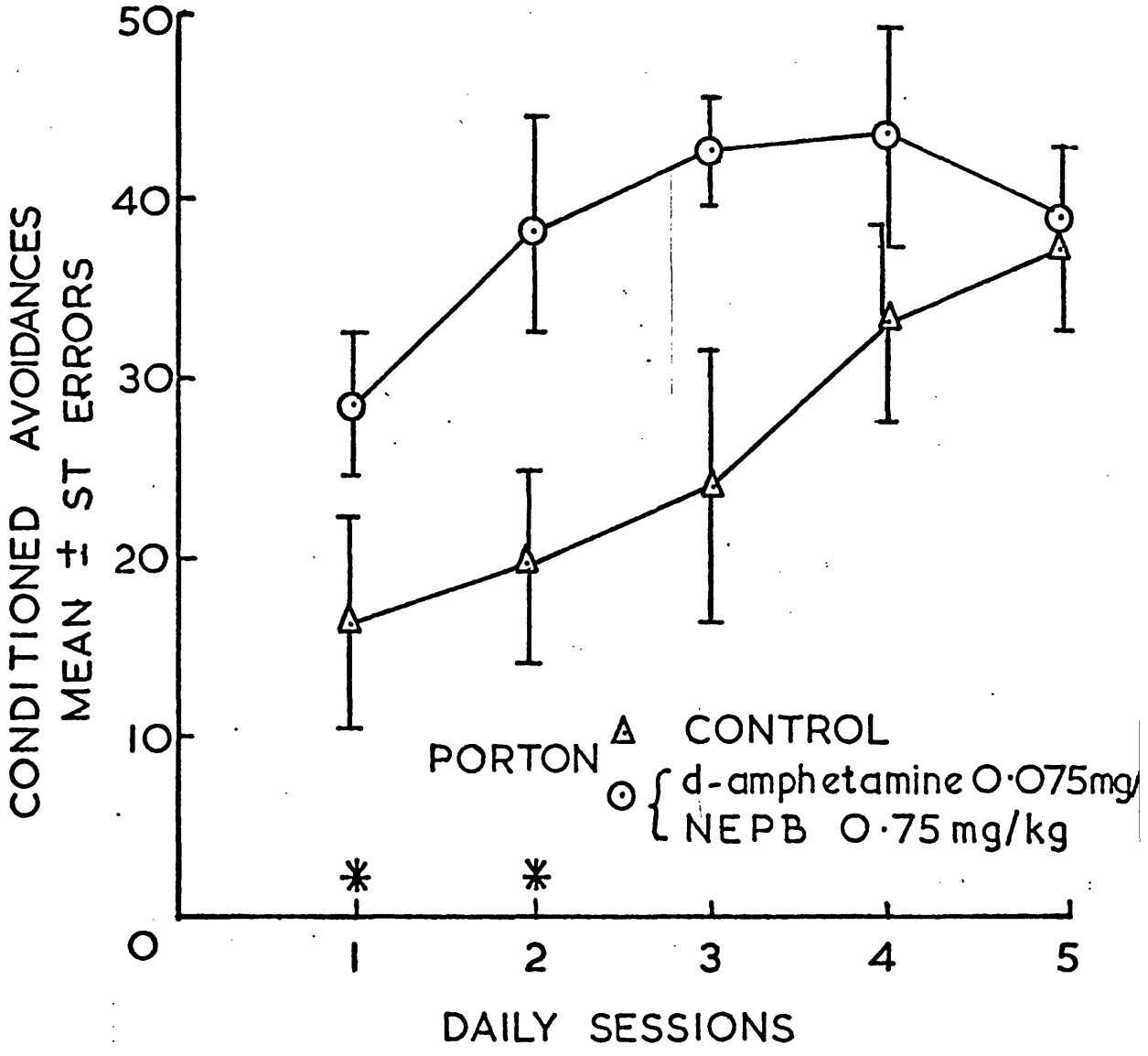


Figure 3-28. Conditioned avoidance learning in the Porton strain rat (10 males) after injection of d-Amphetamine (0.075 mg/kg., s.c.) and NEPB (0.75 mg/kg., i.p.), injected simultaneously, 15 minutes before each of sessions 1 - 4. No drugs were given before session 5.

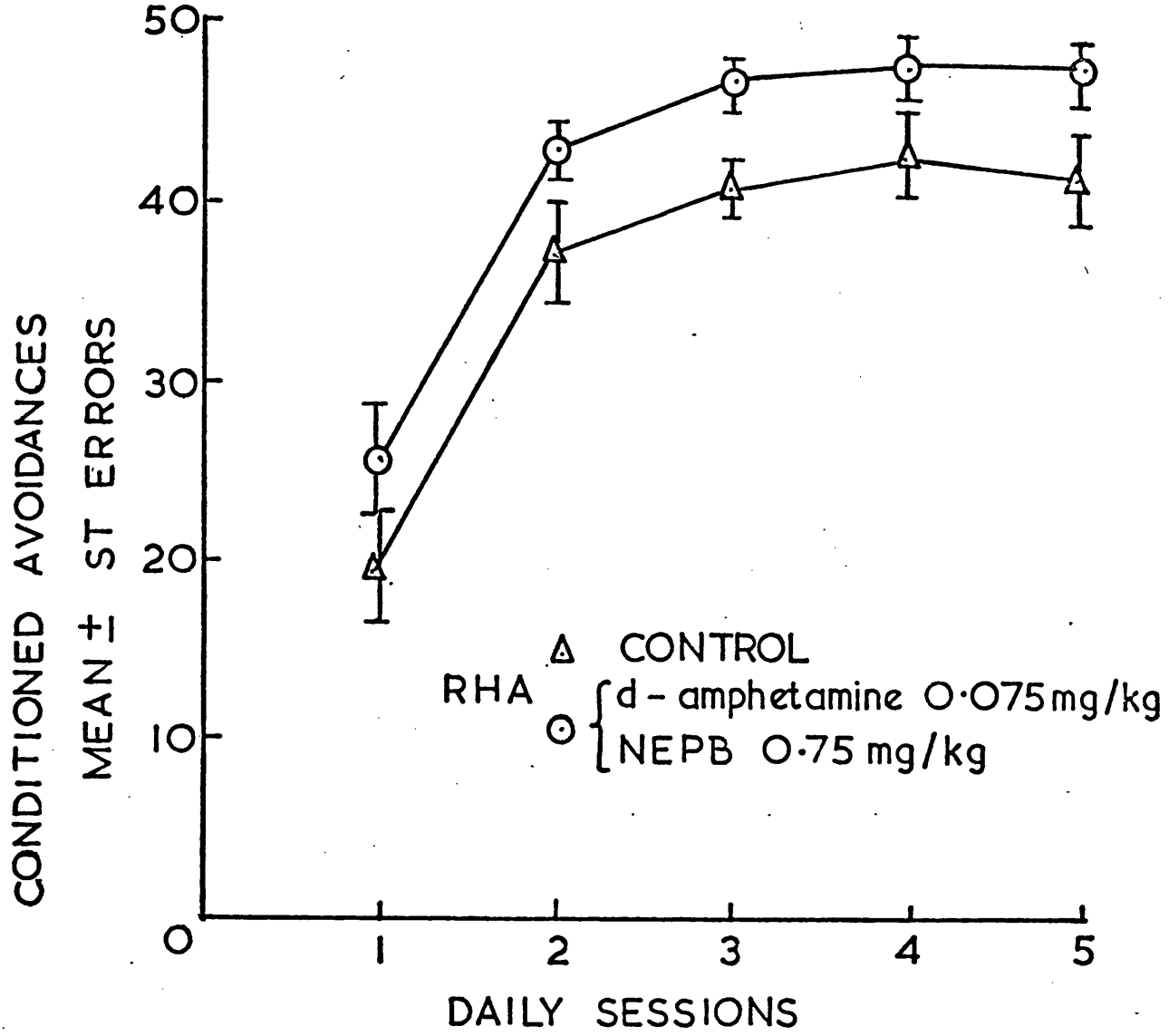


Figure 3-29. Conditioned avoidance learning in the RHA strain rat (10 males) after injection of d-Amphetamine (0.075 mg/kg., s.c.) and NEPB (0.75 mg/kg., i.p.), injected simultaneously, 15 minutes before each of sessions 1 - 4. No drugs were given before session 5. Note that points plotted are failures to make unconditioned responses and avoidance responses.

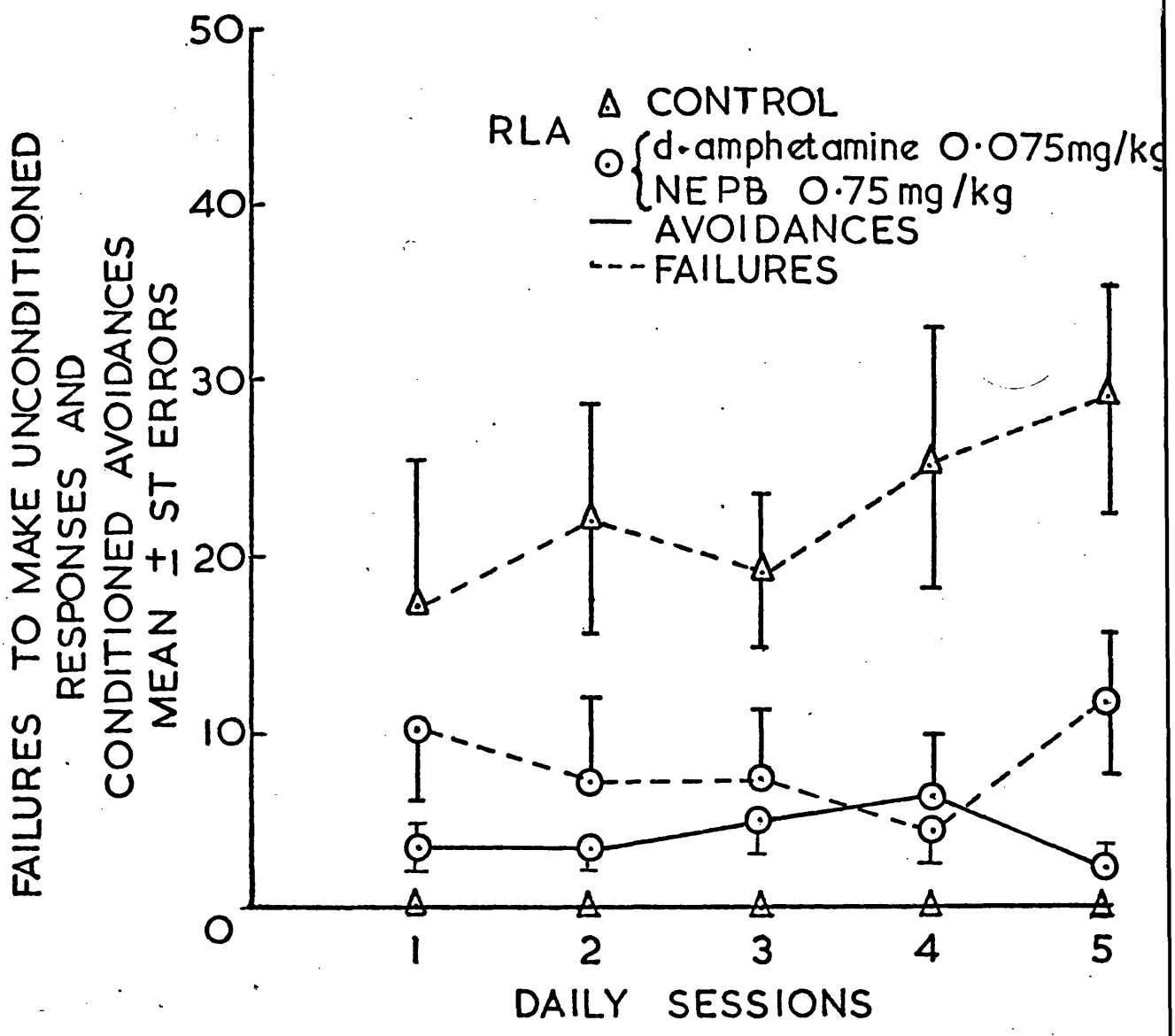


Figure 3-30. Conditioned avoidance learning in the RLA strain rat (10 males) after injection of d-Amphetamine (0.075 mg/kg., s.c.) and NEPB (0.75 mg/kg., i.p.), injected simultaneously, 15 minutes before each of sessions 1 - 4. No drugs were given before session 5. Significant differences are not shown on the graph but are as follows: Failures of unconditioned response, significantly different in sessions 1 - 5. Conditioned avoidances, significantly different in sessions 1 - 4.

3.7 Extinction Experiments

The extinction of condtioned avoidance responding in rats of the Porton and RHA strains was investigated. 8 rats of each strain were trained in sessions of 50 trials each in the shuttlebox, until a criterion of 60% conditioned avoidance was reached. As rats reached this criterion they were removed from the training situation and returned to their home cages until the following day. Extinction training in the shuttlebox was then introduced and consisted of 6 daily sessions of 20 trials each, in which the conditioned stimulus was not followed by shock. Because of the superior conditioning performance exhibited by RHA rats, fewer trials were required to train these rats to the criterion of 60%, than were required for Porton rats. Despite the longer training period of the latter, the RHA strain took much longer to show extinction of the response (Figure 3-31), which was not completely extinguished until the fifth session.

The effects of anti-ACh drugs on this behaviour were also investigated. NEPB (1.0 mg/kg.) or saline (1.0 ml/kg.) was injected, i.p. 15 minutes before each of the extinction sessions. The results of this experiment are shown in Figure 3-32. NEPB significantly delayed extinction of the response in both strains but to the greatest extent in the RHA strain. The Porton strain after NEPB, showed a significantly greater number of avoidances in session 3, but the RHA strain after NEPB did not show significant loss of the avoidance response, even after 6 extinction sessions.

The experiment was repeated and NEPB MeI (1.0 mg/kg.) was injected i.p., 15 minutes before each extinction session. The result of this experiment is shown in Figure 3-33. The drug treated groups showed a tendency to extinguish more quickly than the controls but the effect was not a significant one.

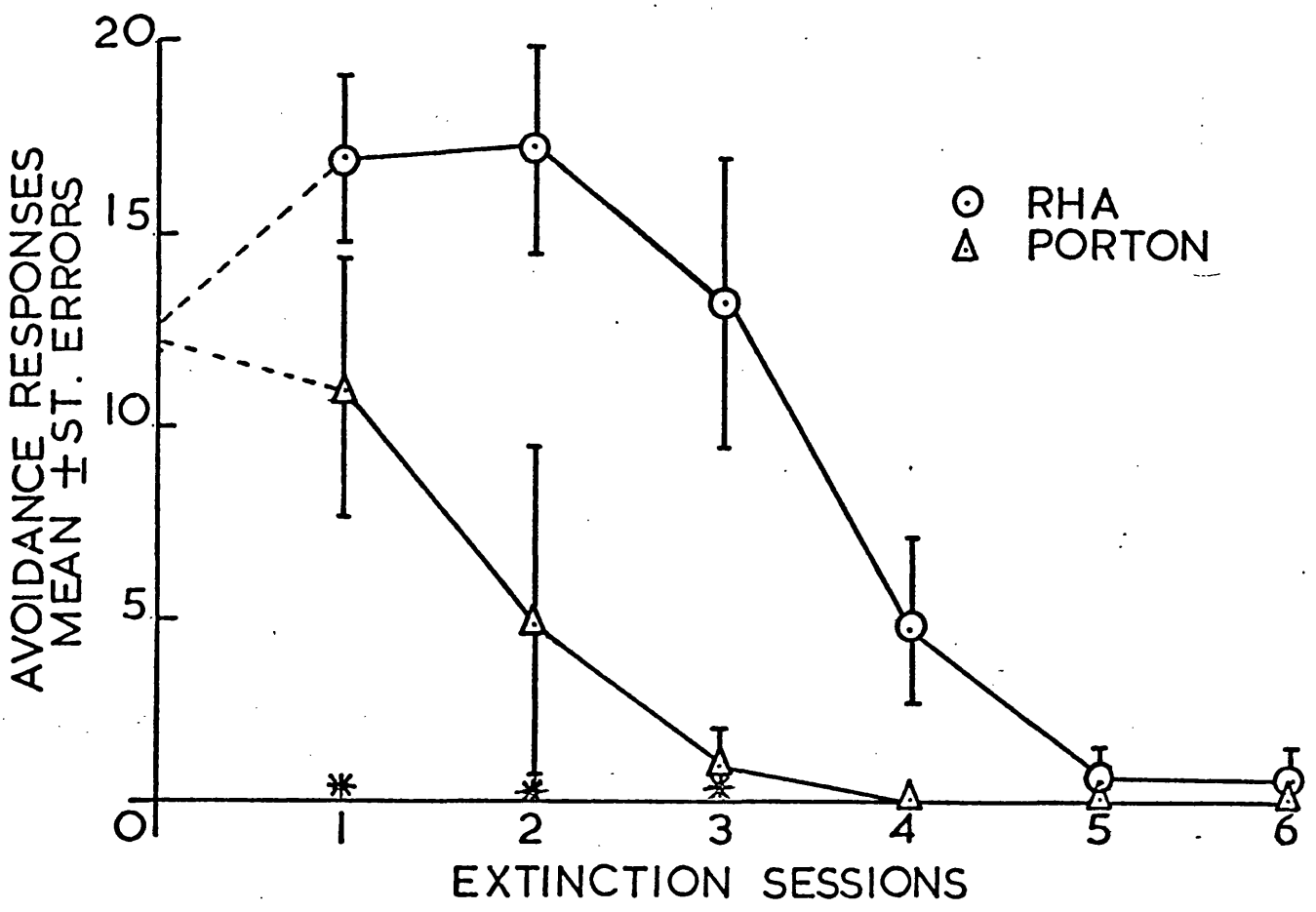


Figure 3-31. Extinction of avoidance responding in the Porton and RHA strains of rat. Subjects (8 males of each strain) previously trained to an avoidance criterion of 60%, given one extinction session in the shuttlebox on each of 6 successive days. An extinction session consisted of 20 trials, in which the conditioned stimulus (tone) was presented without subsequent presentation of shock stimulus.

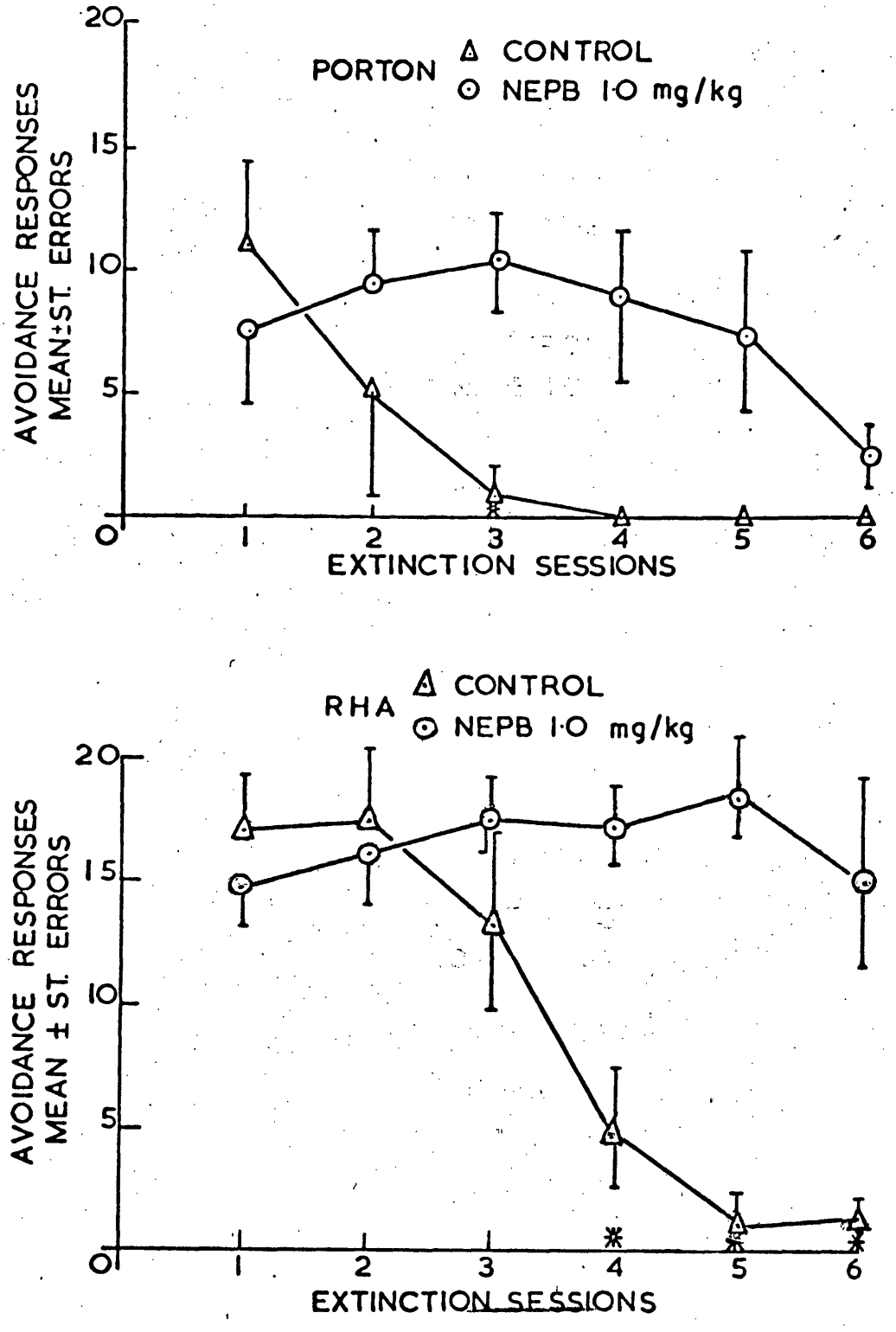


Figure 3-32. Extinction of avoidance responding in the Porton strain (upper graph) and RHA strain (lower graph), after injection of NEPB. Drug was injected (1.0 mg/kg., i.p.) 15 minutes before the beginning of each extinction session. Training similar to that described in the previous figure and in the text.

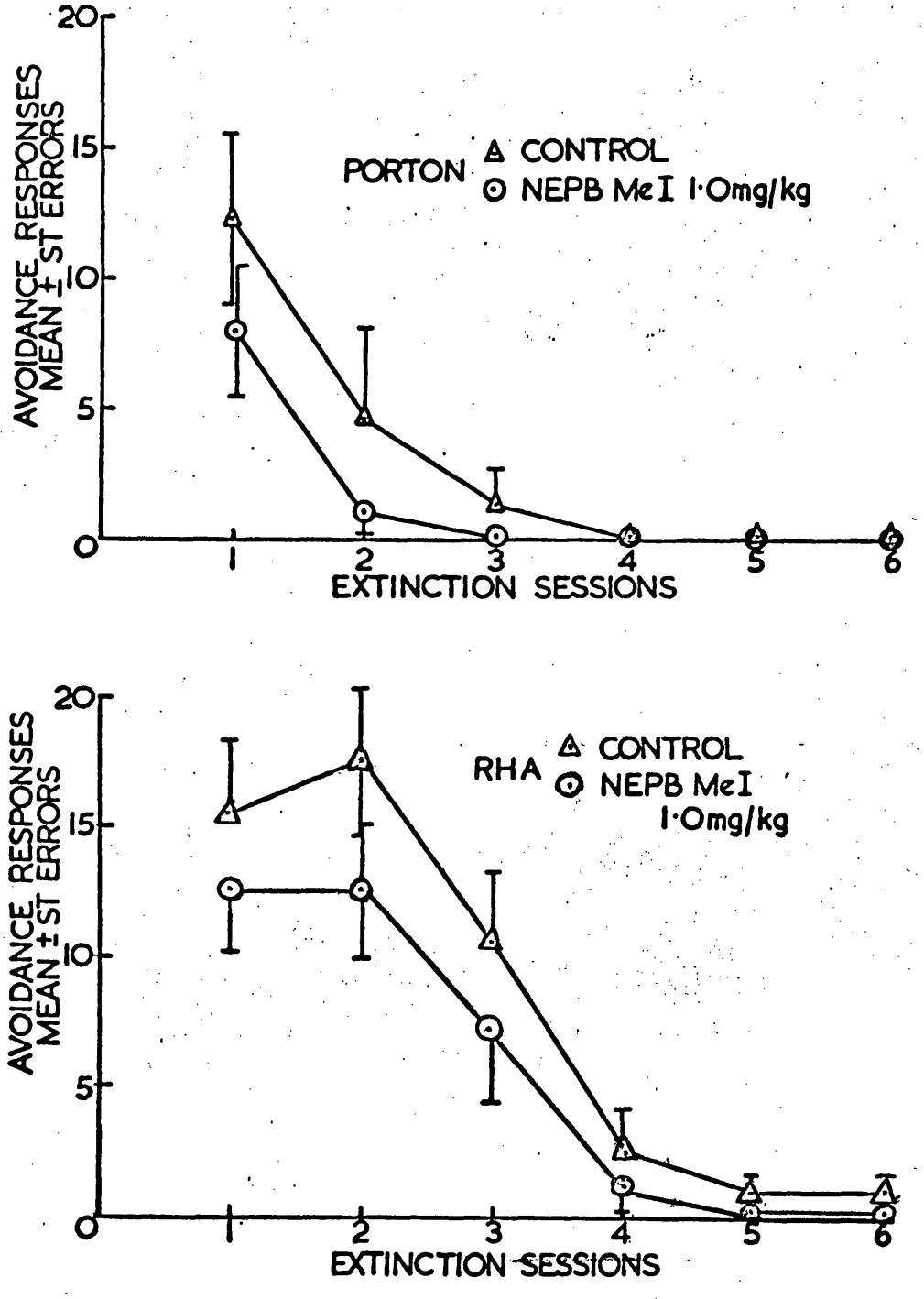


Figure 3-33. Extinction of avoidance responding in the Porton strain (upper graph) and RHA strain (lower graph) after injection of NEPB MeI. Drug was injection (1.0 mg/kg., i.p.) 15 minutes before the beginning of each extinction session. Training similar to that described in the previous two figures.

CHAPTER 4

Spontaneous Activity Experiments

4.1 Materials and Procedure

All the spontaneous activity measurements were made in activity cages supplied by Ugo Basile (Milan). The cages each consisted of a perspex box with opaque sides, having the dimensions 40 cm x 20 cm x 20 cm high. The lid was of transparent perspex permitting overhead illumination. The floor of each box consisted of stainless steel bars wired to an electrical supply in such a way that alternate bars were live and earth when the grid was powered. The current used was below the threshold of feeling for rats, but the presence of the animals' feet making and breaking new electrical circuits between the bars produced changes in the circuit which were recognised and converted into pulses by a control unit. A print-out counter, with a timed printing out facility, recorded these pulses, summed for five minute intervals. Each cage was housed in a sound-attenuating cupboard with a house-light and extractor fan, similar to that described for use with the shuttlebox apparatus (Figure 3-1). The print-out counter was mounted outside the sound-attenuated cupboard.

In all the experiments described the animals to be tested were injected with the drug and immediately placed, singly, into the activity cages, and activity recording began straight away. The animals were not given an opportunity to explore the box before testing so the initial exploratory phase of activity was recorded and included in the experiment. Recording continued for a period of 90 minutes, during which time the animals were left undisturbed. The cages were so designed that it was not possible to supply food and water to the animals during the test, and for this reason it was decided to limit test periods to two hours

so that food and water motivated activity may not appear during the experiment.

Effective recording with this equipment depended upon good electrical contact between feet and bars so it was important that the bars were kept clean. Before each rat was placed into the box the bars were first wiped with a pad moistened with 70% ethanol solution.

Only male rats were used in these experiments because oestrous cycle variation in the spontaneous activity of females is often large and this may have masked drug effects or strain differences, (this author, unpublished observations).

4.2 Normal Spontaneous Activity of the Strains

Procedure

Male rats of the three strains (Porton, n = 36, RHA, n = 22, RLA, n = 27) were placed in the activity boxes for a period of 90 minutes each and activity scores obtained for 5 minute portions of the recording period. Three activity boxes were available for use, so it was convenient to test one member of each strain at each recording session, thus minimising the effects of circadian variation on activity.

Results

To facilitate comparisons of the activity scores, mean activity counts for each strain are displayed with each of the scores for the other two strains. Thus in Figure 4-1, Porton and RHA are compared, Figure 4-2, Porton and RLA and Figure 4-3, RHA and RLA. It can be seen that the RHA strain is the most active one, demonstrating significantly higher activity scores than both Porton and RLA rats in exploratory and locomotor phases of the recordings. There is apparently no difference however, between the activity levels of the Porton and RLA strains.

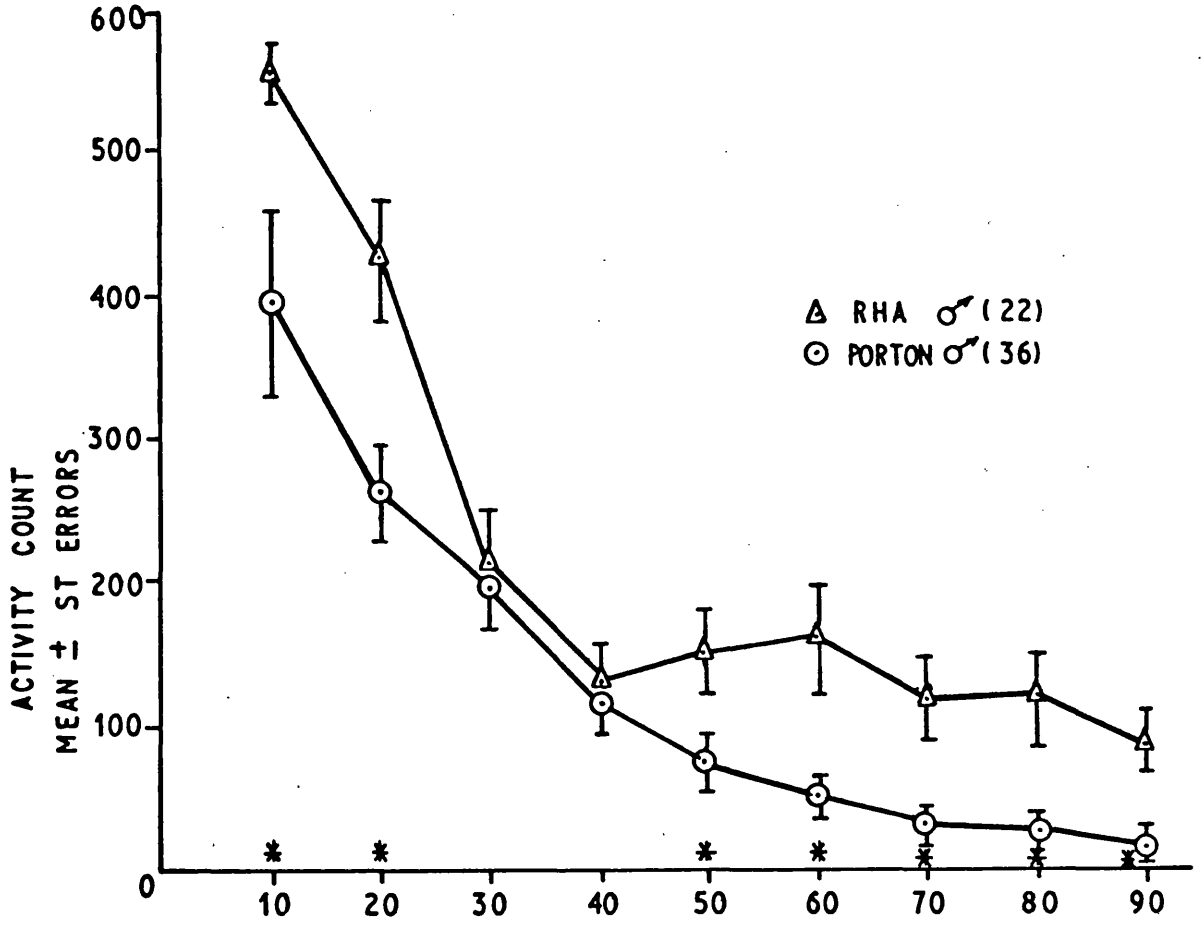


Figure 4-1. Spontaneous activity of RHA strain and Porton strain male rats.

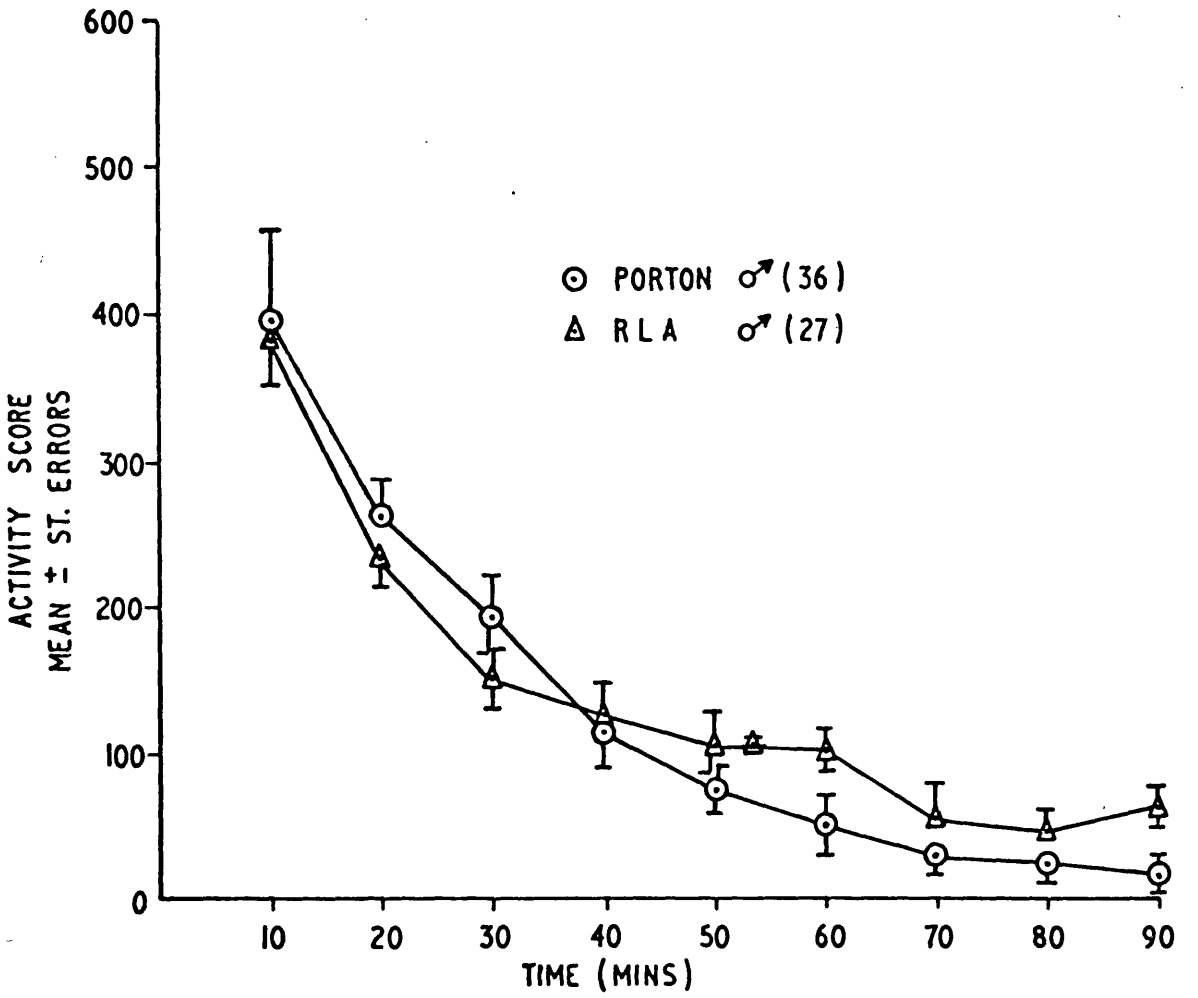


Figure 4-2. Spontaneous activity of RLA strain and Porton strain male rats.

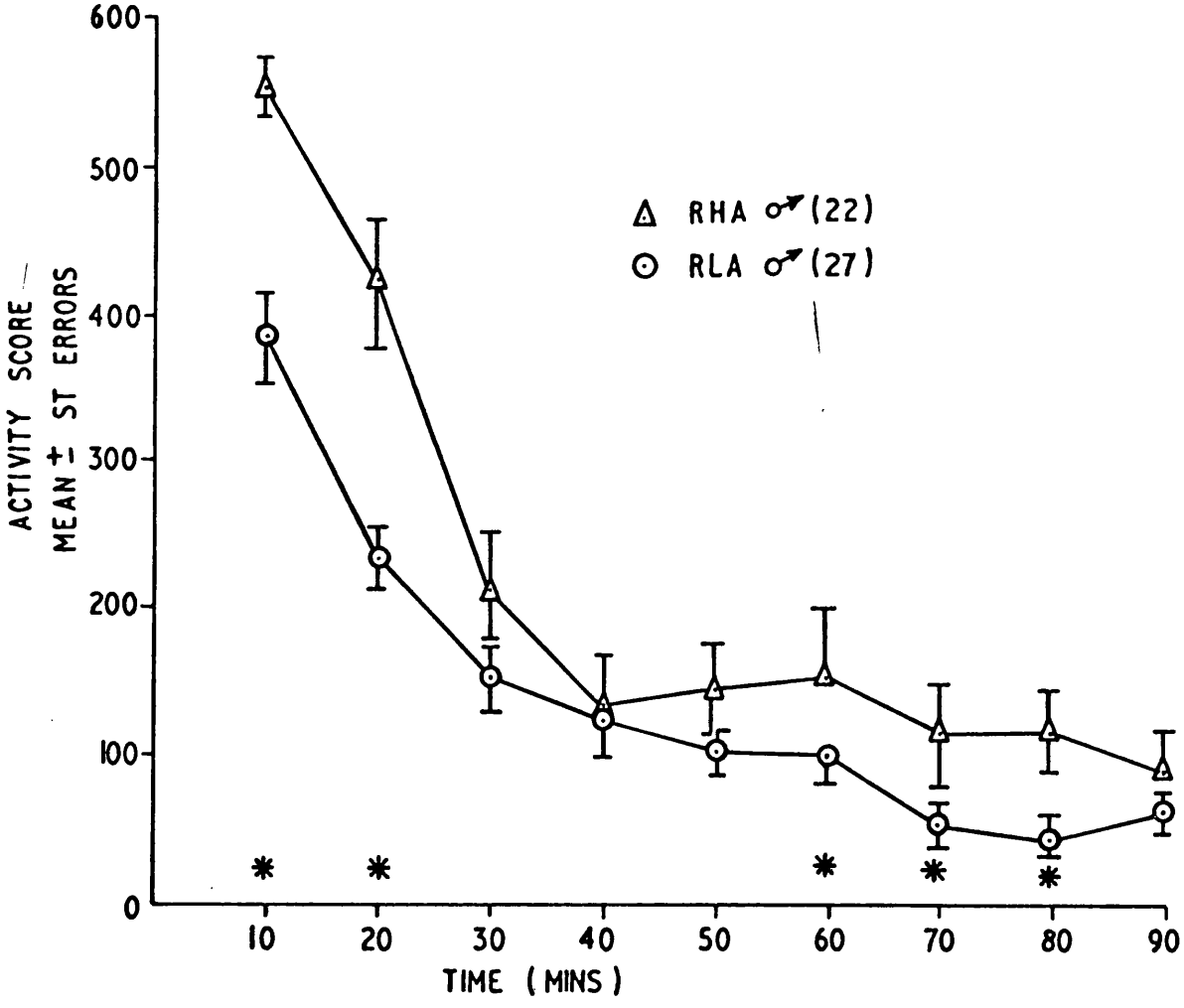


Figure 4-3. Spontaneous activity of RLA strain and RHA strain male rats.

4.3 Spontaneous Activity and Anti-ChE Drugs

Procedure

The effects of two anti-ChE drugs, physostigmine salicylate and pyridostigmine hydrobromide, were examined on the spontaneous activity of the three strains.

Groups of 12 male rats from each of the three strains were injected, s.c., with physostigmine, 0.25 or 0.125 mg/kg., pyridostigmine, 0.125 mg/kg. or saline 1.0 ml/kg., immediately before placing, singly, into the activity boxes. Spontaneous activity was recorded for a period of 90 minutes and print-out totals were taken at 5 minute intervals. Drug treated and saline treated animals were run concurrently whenever possible.

The effects of physostigmine on spontaneous activity are shown in Figure 4-4, Porton strain, Figure 4-5, RHA strain and Figure 4-6, RLA strain.

The effects of physostigmine seen, were mainly depression of exploratory and locomotor activity. The effects were dose dependent and varied in extent between the strains. At the high dose depression of activity was most pronounced during the exploratory phases in all the strains and the duration of this effect varied with strain. The Porton strain rats showed the shortest period of depression, approximately 20 minutes, before exhibiting significantly increased activity for a short period. The RHA rats showed a longer period of depression (30-40 minutes), whilst the RLA rats showed reduced activity, of a less pronounced type, for most of the recording and never showed hyperactivity.

The small dose of physostigmine produced less pronounced effects. The Porton strain showed no changes, but the RHA and RLA showed changed of a smaller magnitude but similar character to those seen after the high dose.

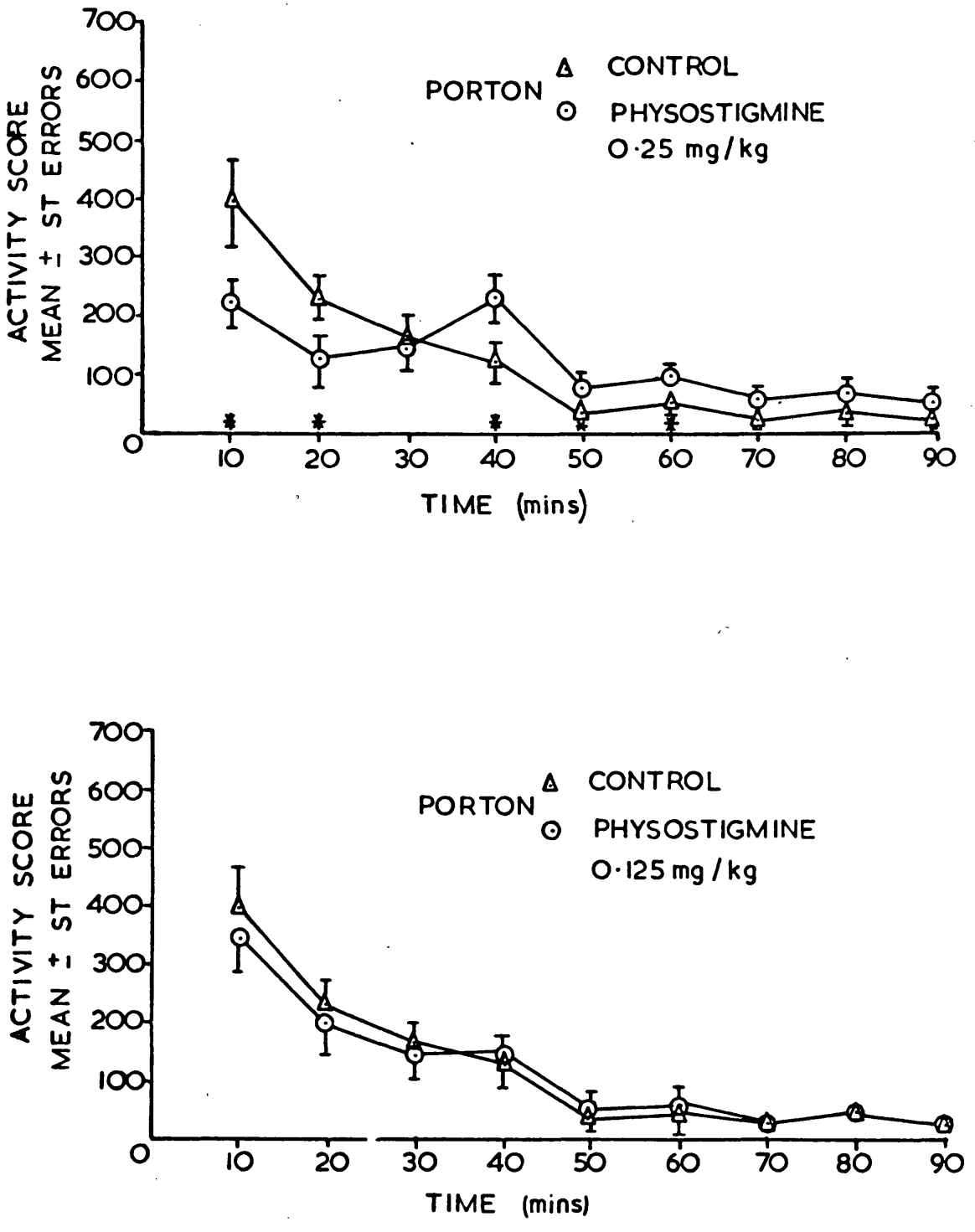


Figure 4-4. Spontaneous activity in Porton strain rats (12 males) after injection of Physostigmine, 0.25 mg/kg., (upper graph) and 0.125 mg/kg/ (lower graph). Drugs injected (s.c.) immediately before placing in activity cages.

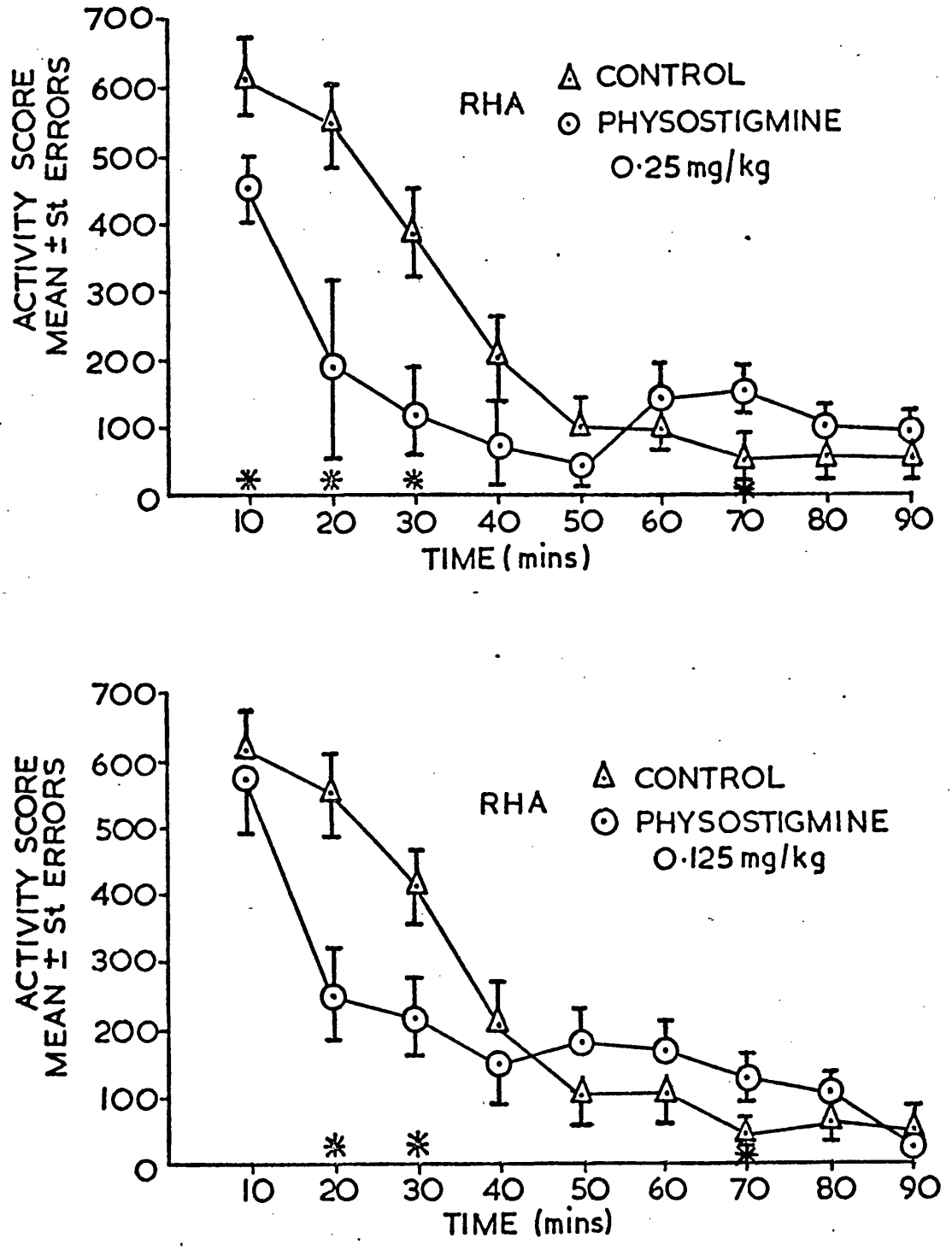


Figure 4-5. Spontaneous activity in RHA strain rats (12 males) after injection of Physostigmine, 0.25 mg/kg. (upper graph) and 0.125 mg/kg. (lower graph). Drugs injected (s.c.) immediately before placing in activity cages.

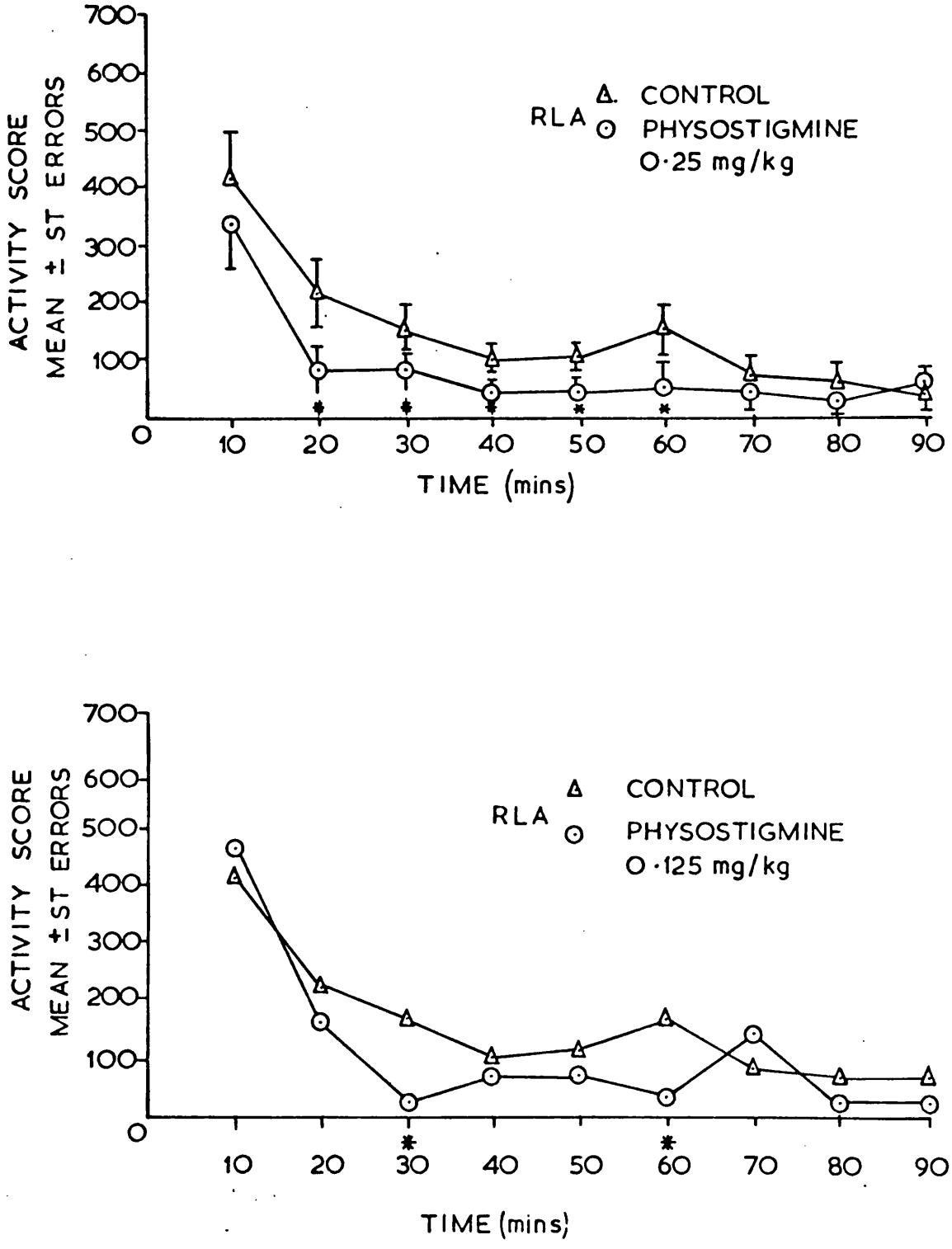


Figure 4-6. Spontaneous activity in RLA strain rats (12 males) after injection of Physostigmine 0.25 mg/kg. (upper graph) and 0.125 mg/kg. (lower graph). Drugs injected (s.c.) immediately before placing in activity cages.

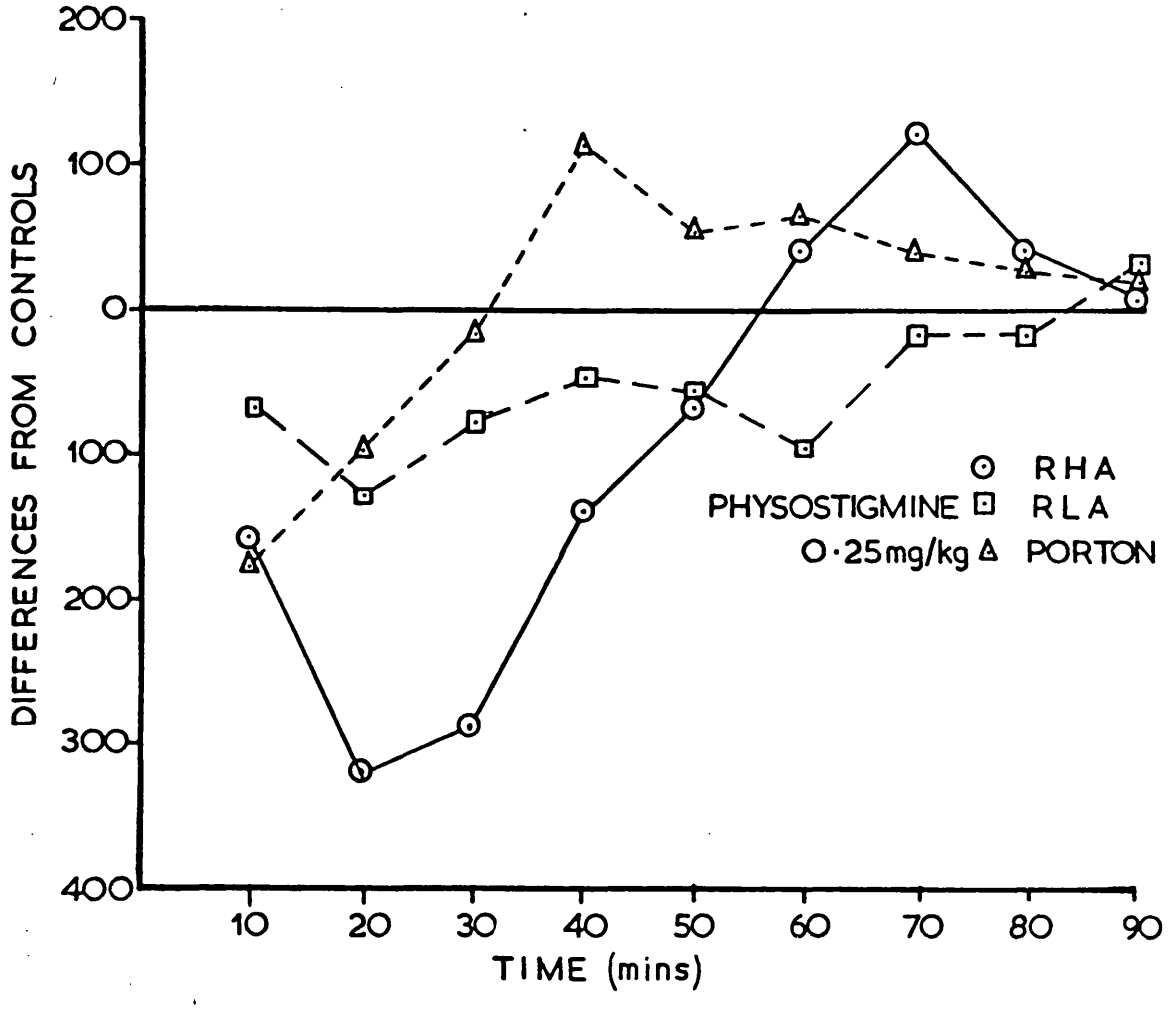


Figure 4-7. Spontaneous activity after Physostigmine, 0.25 mg/kg., in the 3 strains. Difference from controls (mean activity scores taken from Figures 4-4, 4-5 and 4-6).

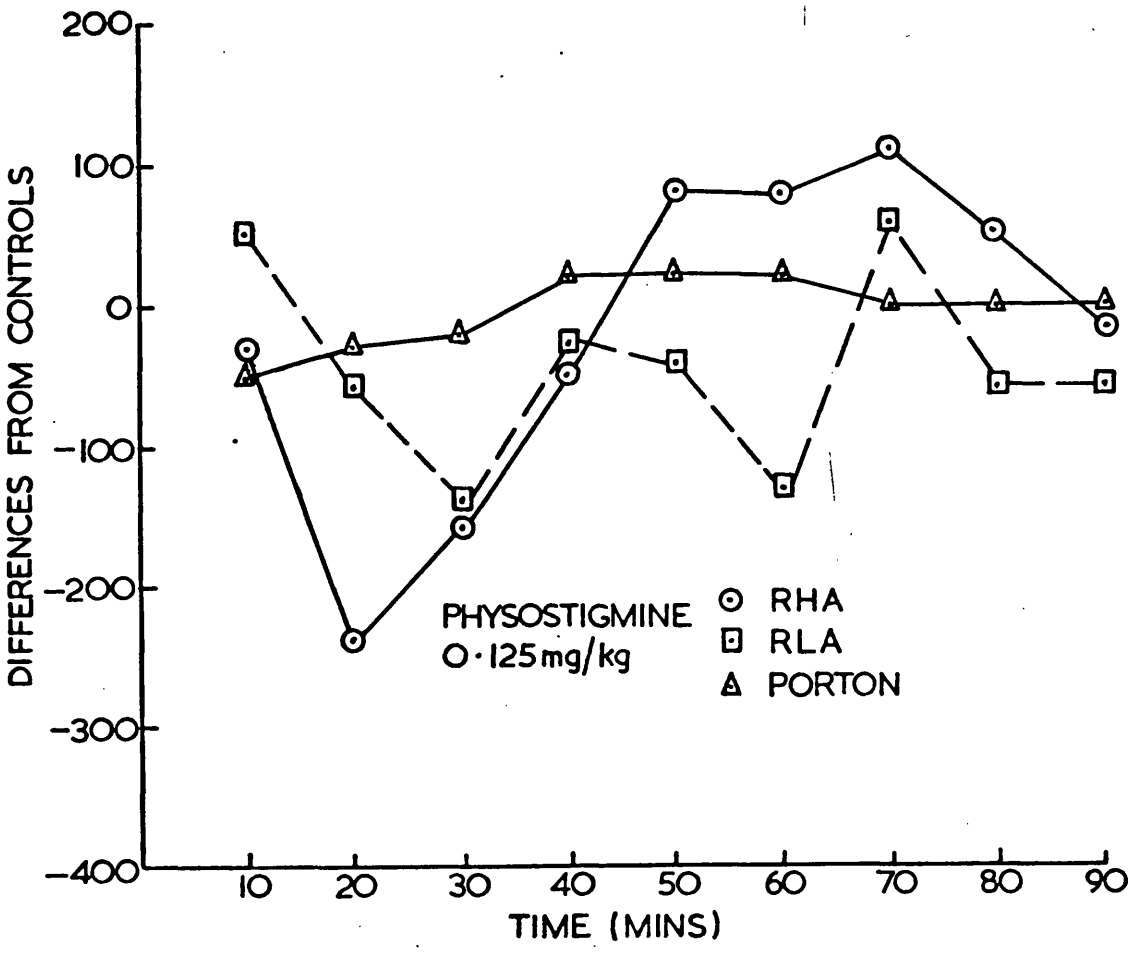


Figure 4-8. Spontaneous activity after Physostigmine, 0.125 mg/kg., in the 3 strains. Differences from controls. (mean activity scores taken from Figures 4-4, 4-5, and 4-6).

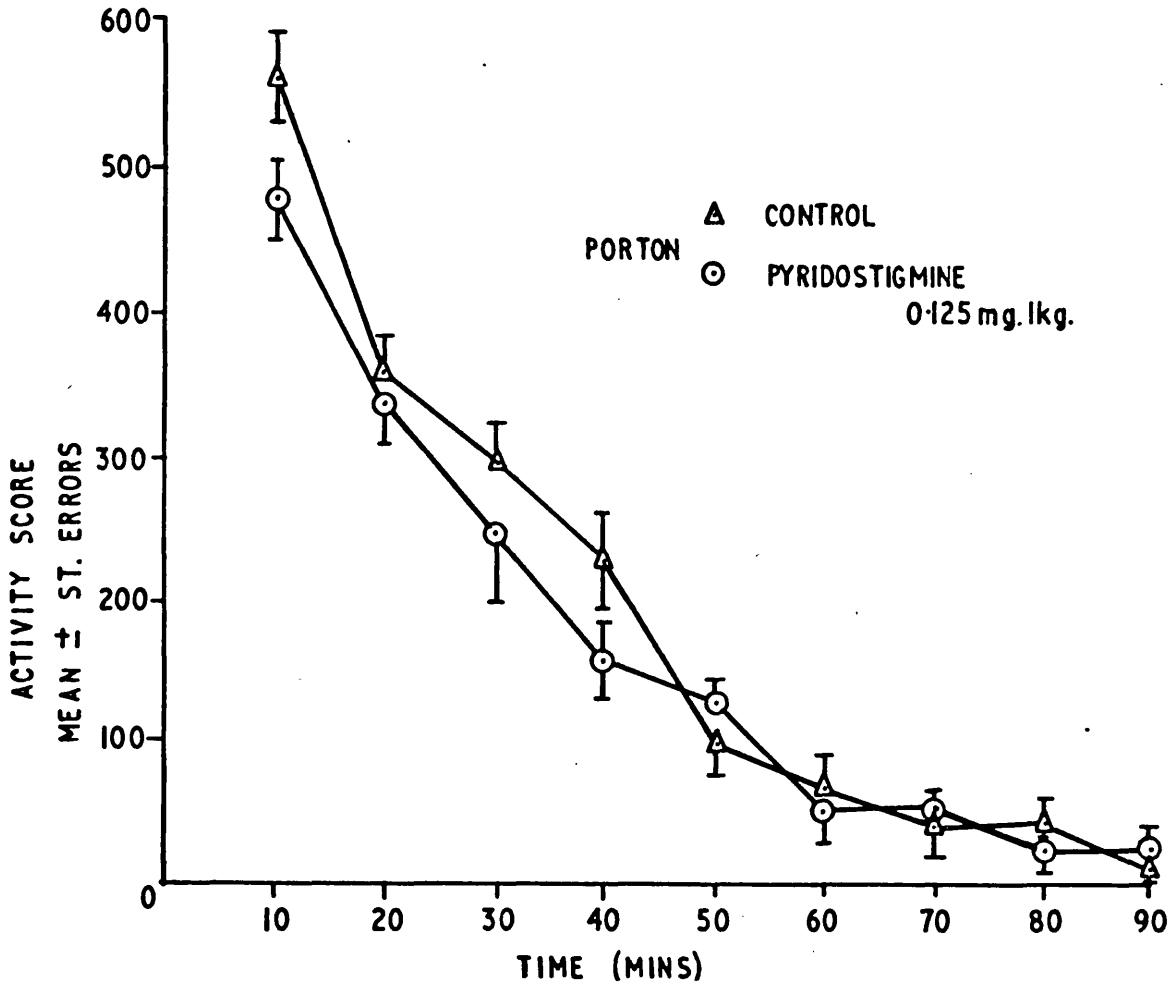


Figure 4-9. Spontaneous activity of Proton strain males injected (s.c.) with Pyridostigmine (0.125 mg/kg.) immediately before placing in recording cages.

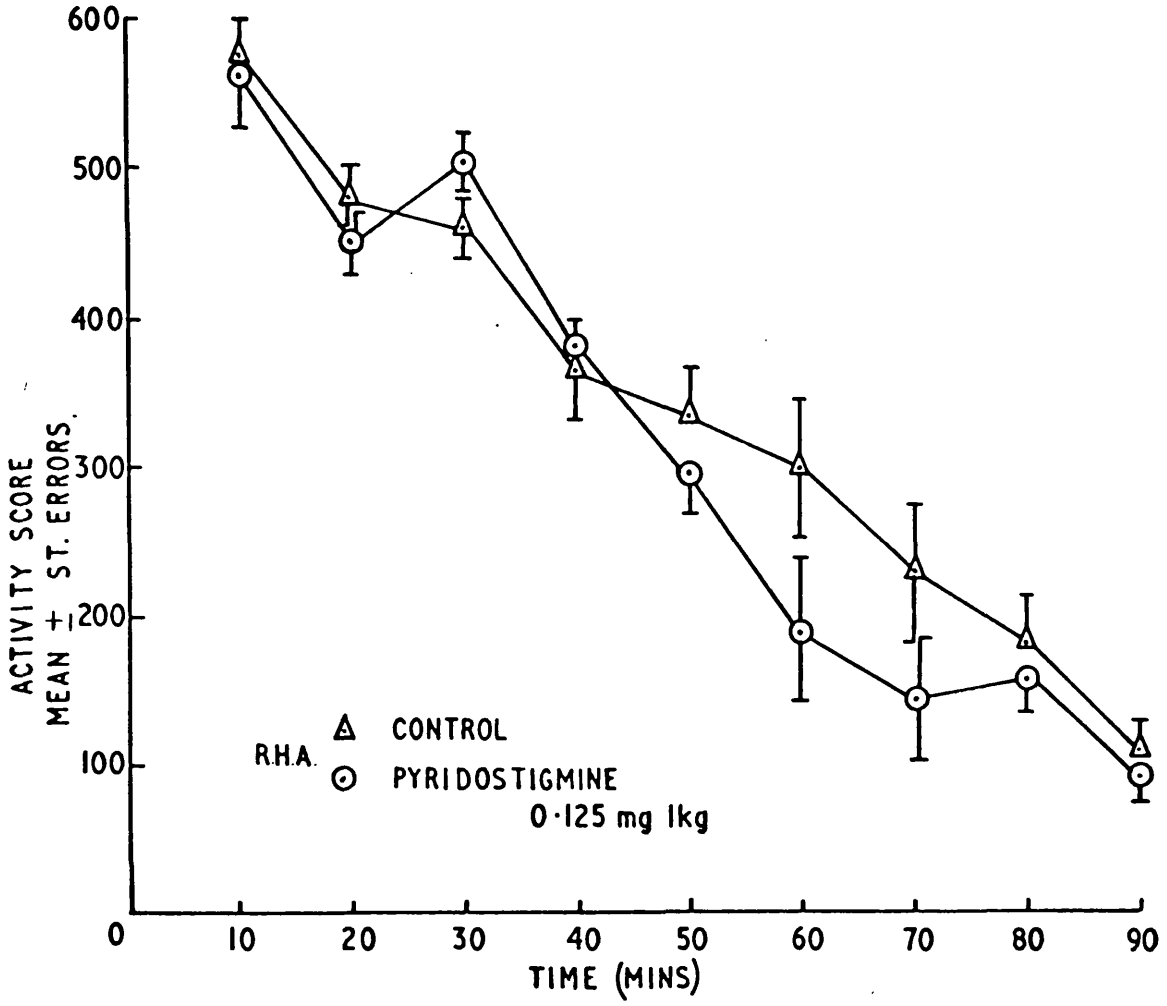


Figure 4-10. Spontaneous activity of RHA strain males injected (s.c.) with Pyridostigmine (0.125 mg/kg.), immediately before placing in recording cages.

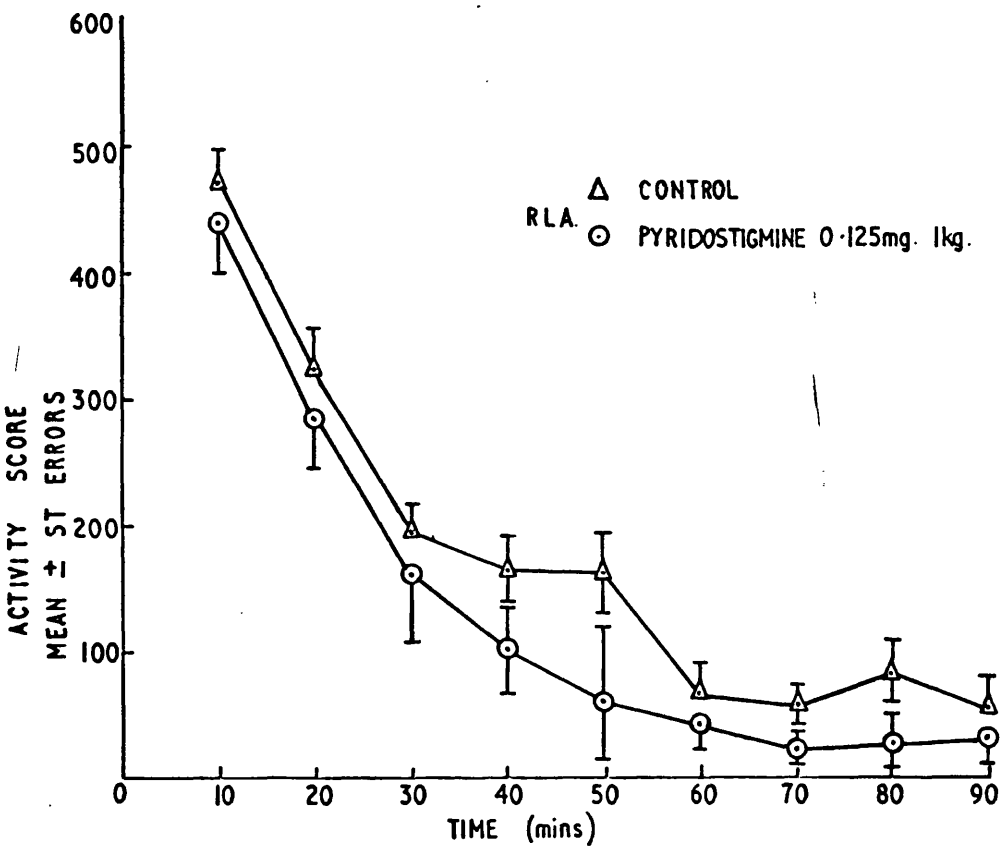


Figure 4-11. Spontaneous activity of RLA strain males injected (s.c.) with Pyridostigmine (0.125 mg/kg.) immediately before placing in recording cages.

Since each strain has a characteristic activity level of its own, plots of activity in which differences from control are shown (Figures 4-7 and 4-8) to facilitate examination of the strain differences.

Spontaneous activity after pyridostigmine is shown in Figures 4-9, Porton strain, Figure 4-10, RHA strain and Figure 4-11, RLA strain. Although some evidence for depression is seen in the RHA and RLA rats after pyridostigmine, at no time did the differences reach statistical significance.

4.4 Spontaneous Activity and Anti-ACh Drugs

Procedure

The effects of two anti-ACh drugs, NEPB and NEPB MeI, on the spontaneous activity of the three strains were examined. The procedure was similar to that used in the physostigmine experiments, and the drugs were injected (i.p.), immediately before placing in the activity boxes, as follows; NEPB 2.0 and 1.0 mg/kg., NEPB MeI 1.0 mg/kg.

The effects of NEPB and NEPB MeI are shown in Figure 4-12, Porton strain, Figure 4-13, RHA strain and Figure 4-14, RLA strain.

Hyperactivity was seen after both doses of NEPB in all the strains, but a dose dependent effect was only seen with the RHA strain. Thus the RHA rats had increased activity scores in the exploratory and locomotor phases at the high dose, but showed hyperactivity of a minor type and only for the initial count of the recording at the low dose. The Porton strain showed slight hyperactivity in the initial part of the exploratory phase after both doses used but no changes in activity after this. The RLA strain, however, showed pronounced hyperactivity throughout the recording period, and to approximately the same degree, after each dose.

Differences from control scores, for these experiments, are shown in Figures 4-15 and 4-16.

The effects of NEPB MeI on spontaneous activity was, at first,

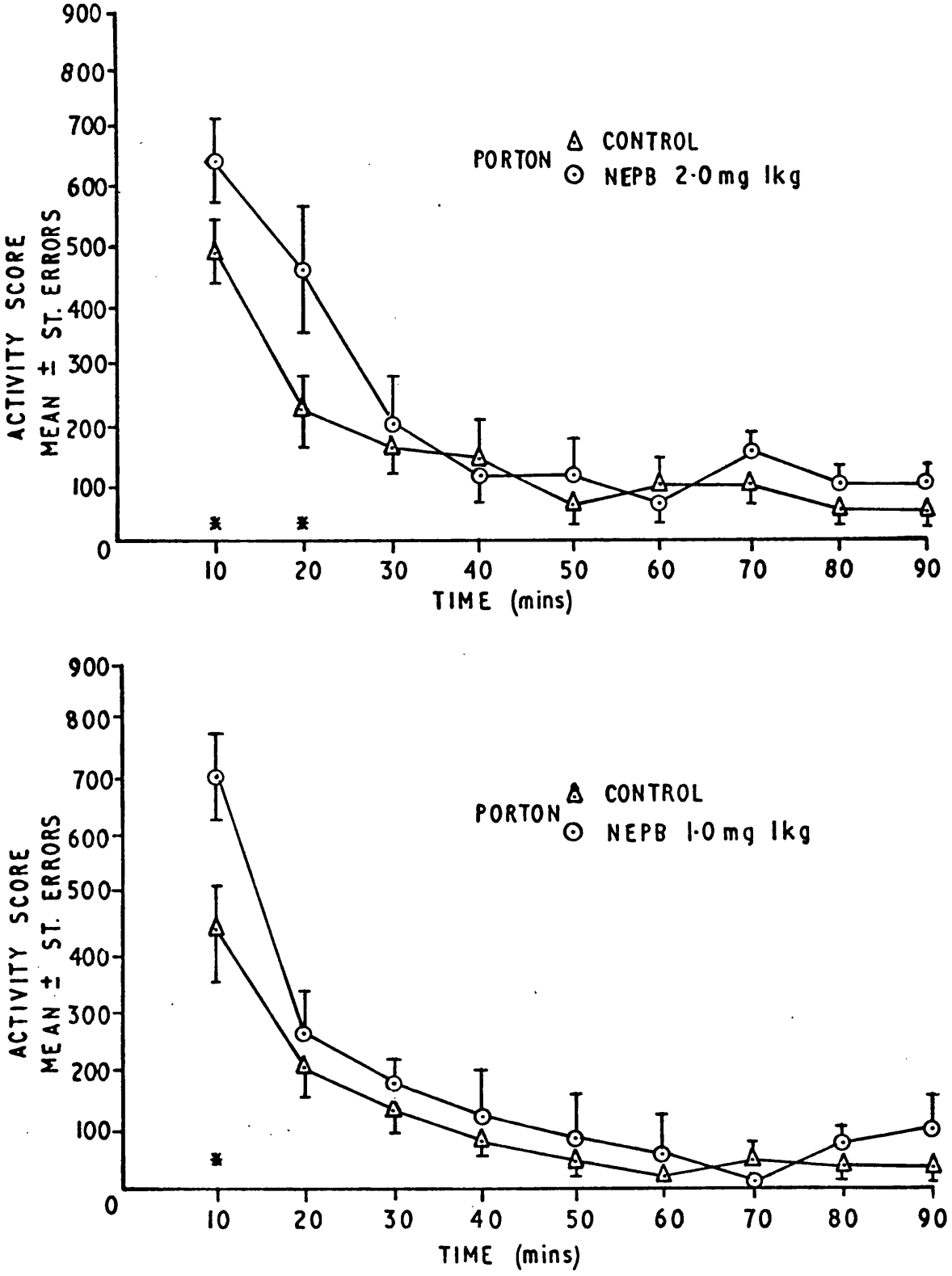
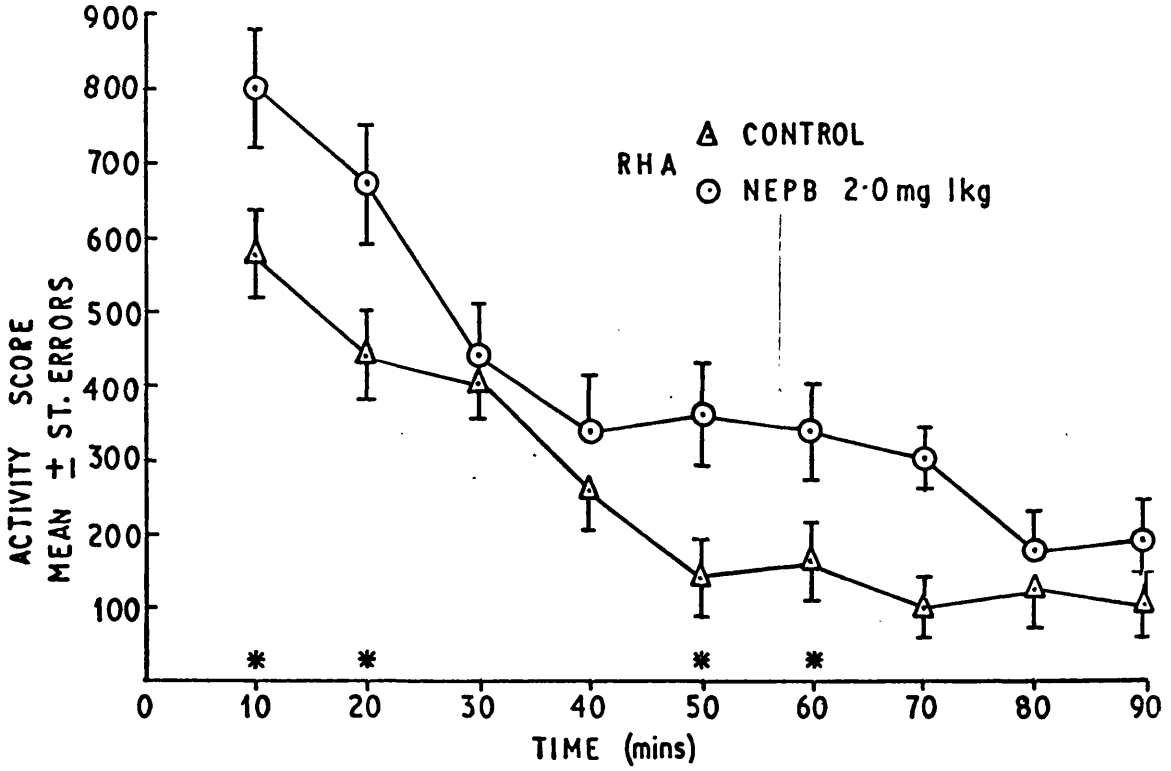


Figure 4-12. Spontaneous activity of Porton strain males injected (i.p.) with NEPB (2.0 mg/kg., upper graph and 1.0 mg/kg., lower graph) immediately before placing in recording cages.



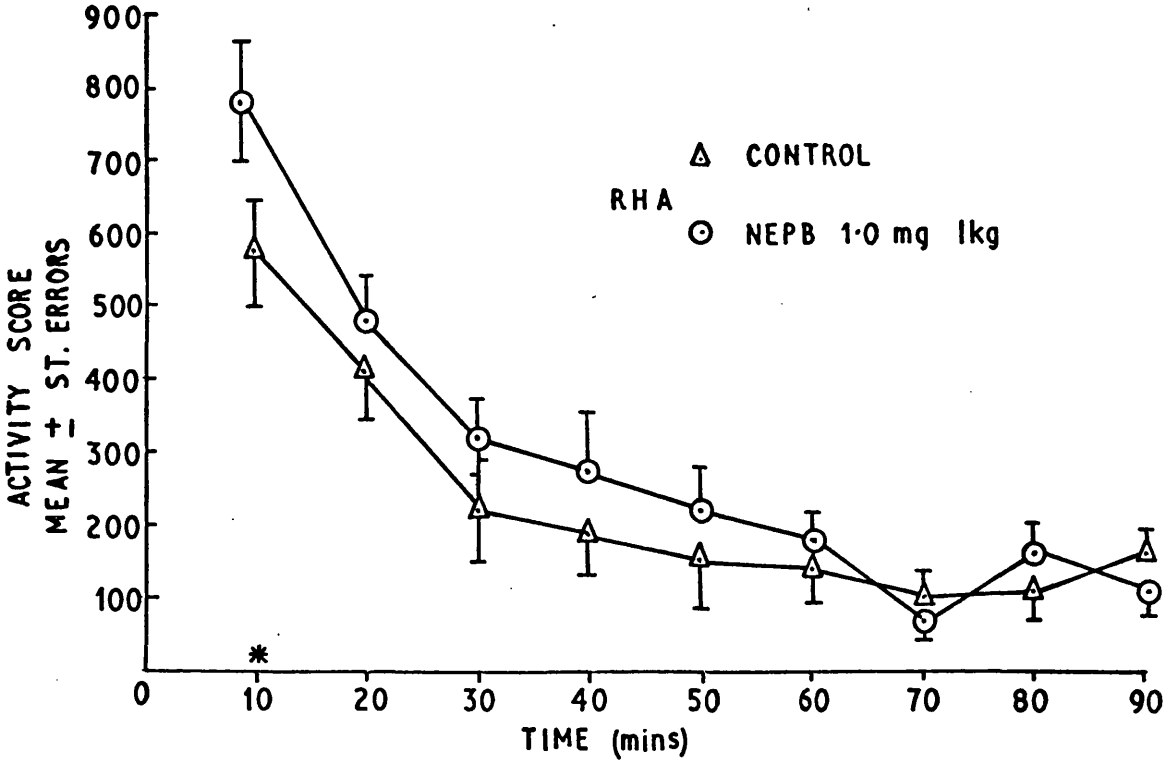


Figure 4-13. Spontaneous activity of RHA strain males injected (i.p.) with NEPB (2.0 mg/kg., upper graph and 1.0 mg/kg., lower graph) immediately before placing in recording cages.

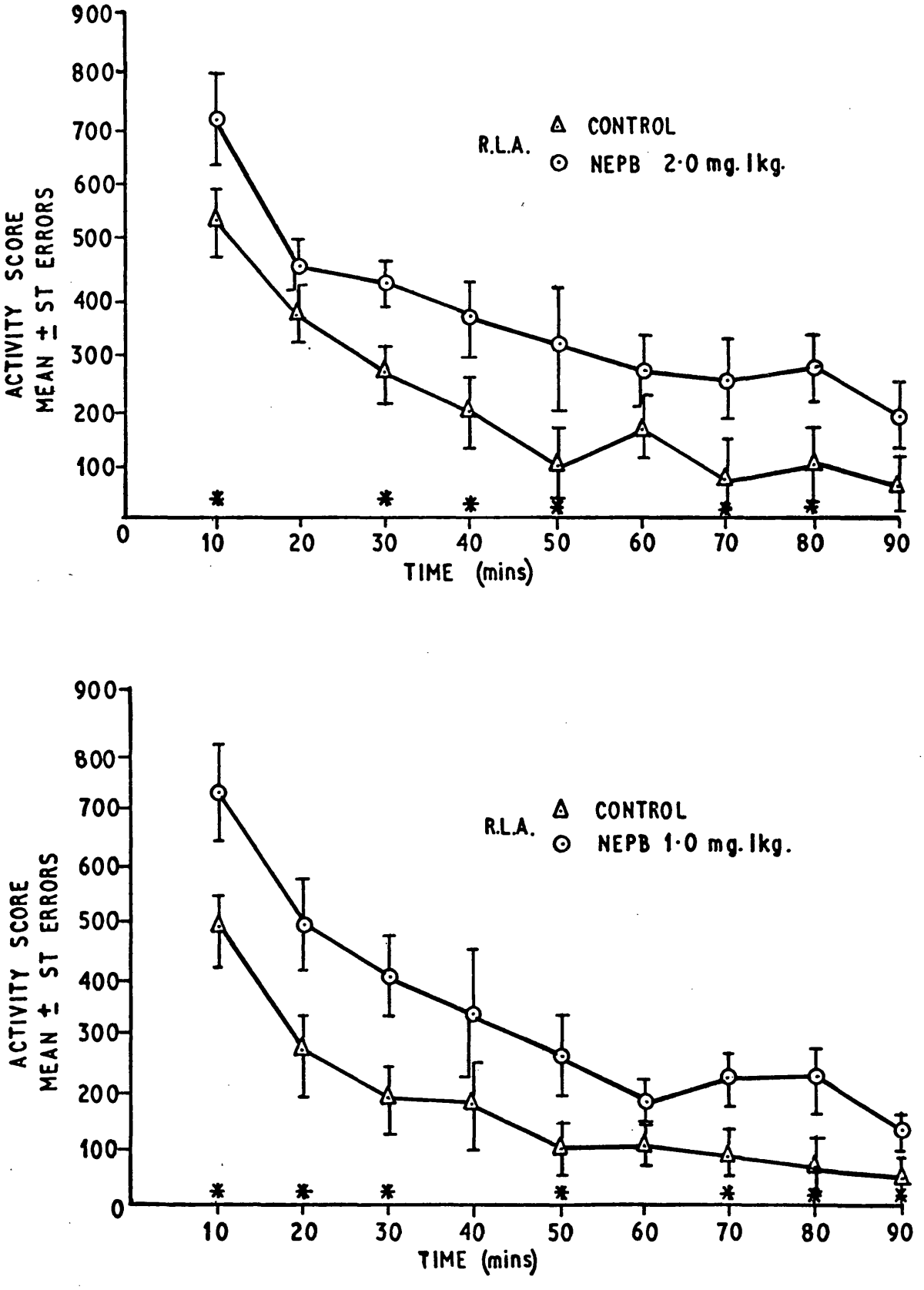


Figure 4-14. Spontaneous activity of RLA strain males injected (i.p.) with NEPB (2.0 mg/kg., upper graph and 1.0 mg/kg., lower graph) immediately before placing in recording cages.

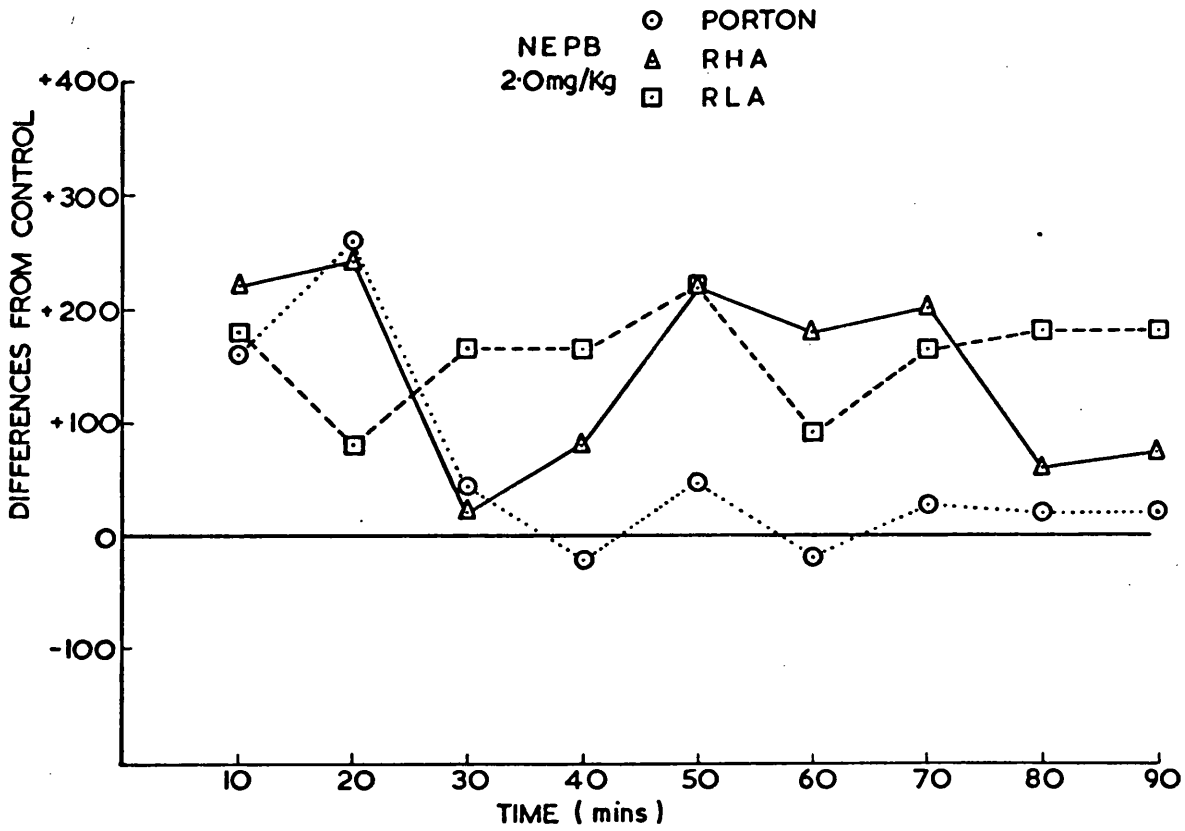


Figure 4-15. Spontaneous activity after NEPB (2.0 mg/kg.), in the 3 strains. Differences from controls (mean activity scores taken from Figures 4-12, 4-13 and 4-14).

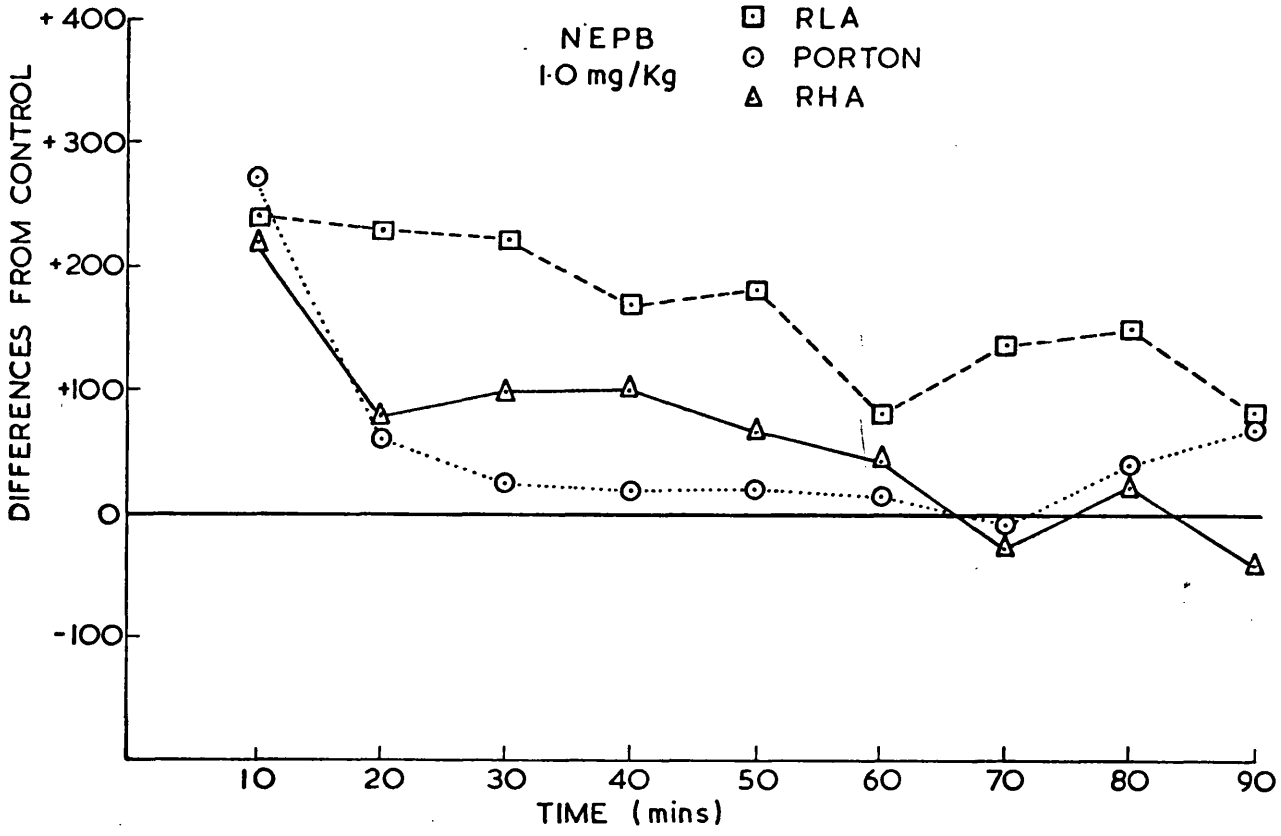


Figure 4-16. Spontaneous activity after NEPB (1.0 mg/kg.), in the 3 strains. Differences from controls (mean activity scores taken from Figures 4-12, 4-13 and 4-14).

(Porton strain, Figure 4-17, upper graph) seen to be one of reduced activity, but this was shown to be the result of a recording artifact caused by poor electrical contact. A second experiment using the Porton strain and a similar dose of NEPB MeI, showed no differences from control, after the grid floor bars had been wiped with saline gel. It was concluded that the drug caused changes in skin resistance which were responsible for poor recording but that the application of saline gel reversed this effect. (The experiment performed with Porton rats and NEPB was repeated using saline gel. Similar recordings were obtained to those recorded without saline gel, showing that NEPB did not produce this effect to a significant degree). The remaining experiments performed to examine the effects of NEPB MeI on spontaneous activity in the strains were performed with saline gel on the grid bars.

The effects of NEPB MeI on the spontaneous activity of RHA and RLA rats, is shown in Figure 4-18. No significant changes were seen.

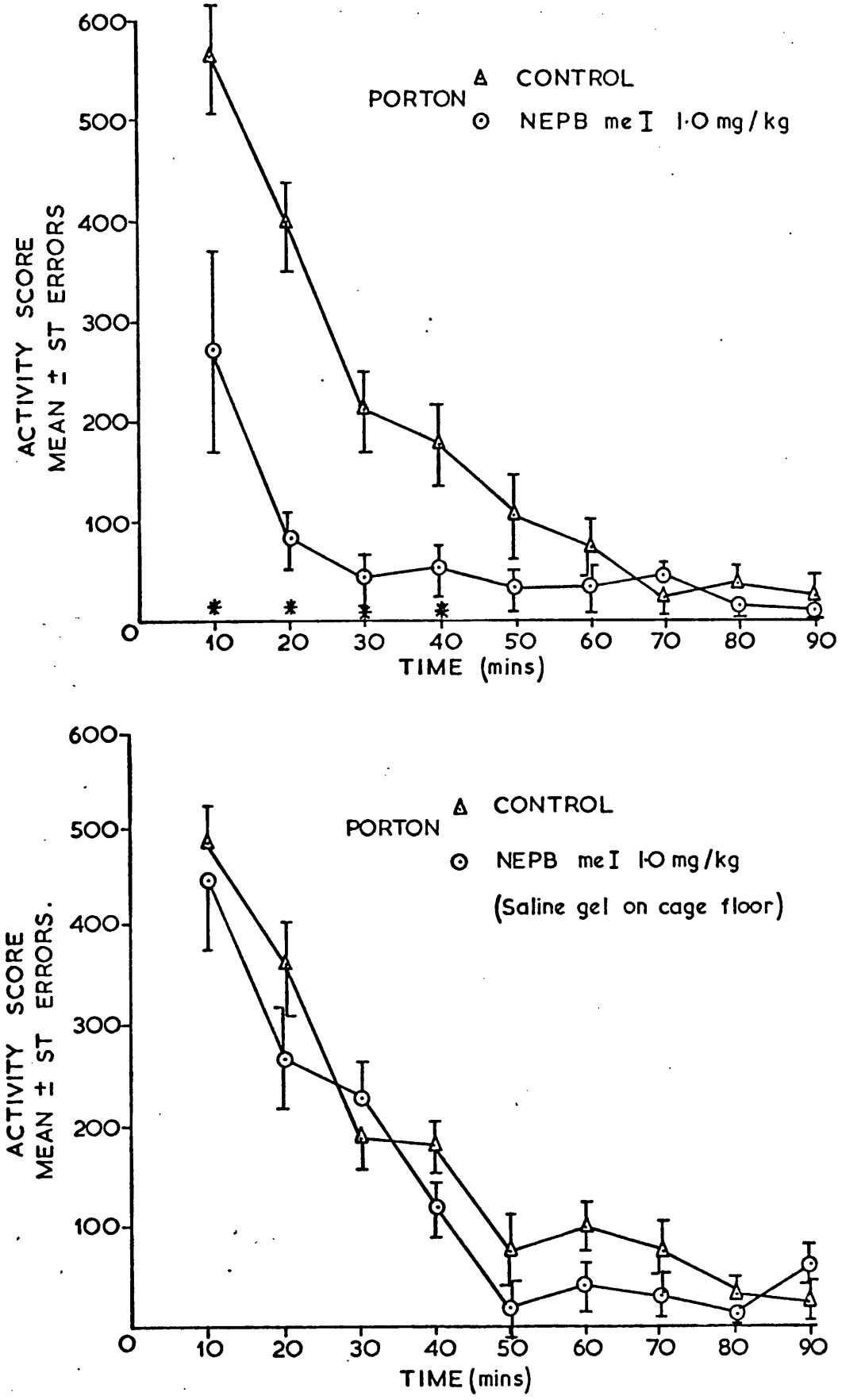


Figure 4-17. Spontaneous activity in Porton strain rats (8 males) after injection of NEPB MeI, 1.0 mg/kg. Drug injected (i.p.), immediately before placing into activity cage. Upper graph, normal procedure and lower graph, activity cage floor wiped with saline gel before use.

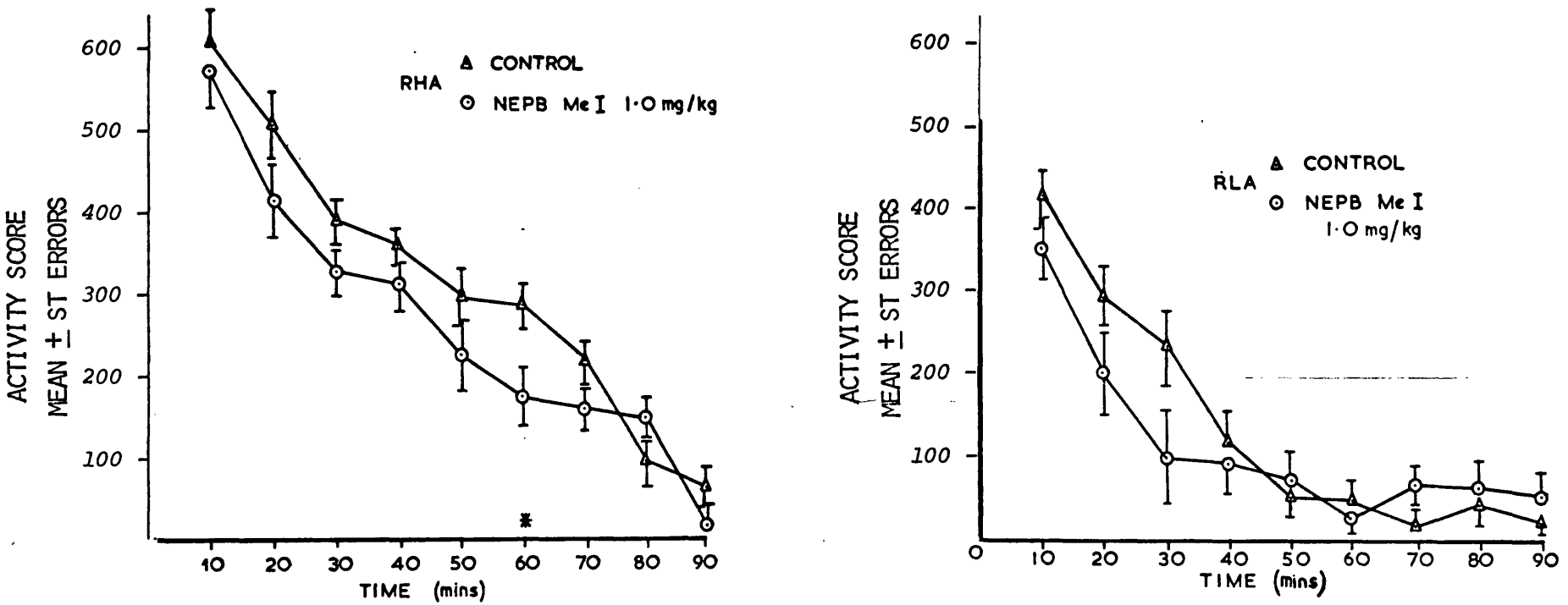


Figure 4-18. Spontaneous activity in RHA and RLA strain rats (10 males) after injection of NEPB MeI, 1.0 mg/kg. Drug injected (i.p.) immediately before placing in activity cage. Left-hand graph, RHA rats, right-hand graph, RLA rats. Cage bars wiped with saline gel. (See previous figure and text).

4.5 Spontaneous Activity and d-Amphetamine

Procedure

The effects of d-Amphetamine on spontaneous activity in the three strains was examined. A similar procedure was used as for the other spontaneous activity experiments and d-Amphetamine (0.1 mg/kg.) or saline 10. ml/kg., was injected, s.c., immediately before placing the animals singly, into the activity boxes.

The effects of spontaneous activity are shown in Figure 4-19. Porton strain, Figure 4-20, RHA strain and Figure 4-21, RLA strain. None of the strains showed initial changes in activity but all strains showed hyperactivity at later stages. Different effects were seen between the strains, the Porton rats showing slightly increased activity, but both Roman strains showing hyperactivity, of a pronounced character. Differences from control scores are plotted for these experiments, Figure 4-22 from which it can be seen that the RLA strain rats showed the greatest increases in activity.

4.6 Spontaneous Activity after Combined Treatment with Anti-ACh and Adrenergic Drugs

Procedure

The effect on spontaneous activity, of giving a combination of NEPB and d-Amphetamine at doses, which given separately produce no significant changes in activity, was investigated in the Porton strain. It was discovered in pilot experiments, that NEPB at a dose of 0.75 mg/kg., and d-Amphetamine at a dose of 0.075 mg/kg., did not significantly change spontaneous activity of the Porton rat and were therefore chosen for use in this study.

NEPB (0.75 mg/kg.) was injected, i.p., followed immediately by d-Amphetamine (0.075 mg/kg.), injected, s.c., to a group of 10 male, Porton rats and their activity was compared with that of similar groups,

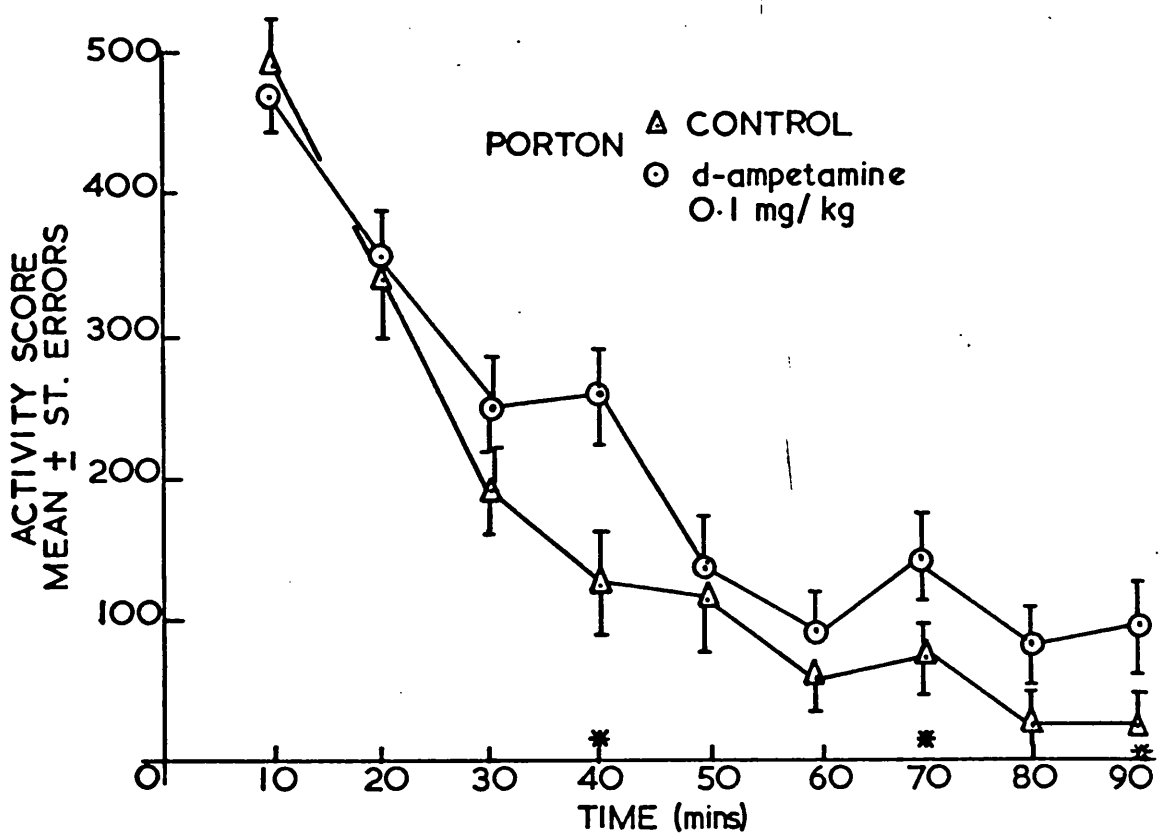


Figure 4-19. Spontaneous activity in Porton strain males injected (s.c.) with d-Amphetamine (0.1 mg/kg.), immediately before placing in recording cages.

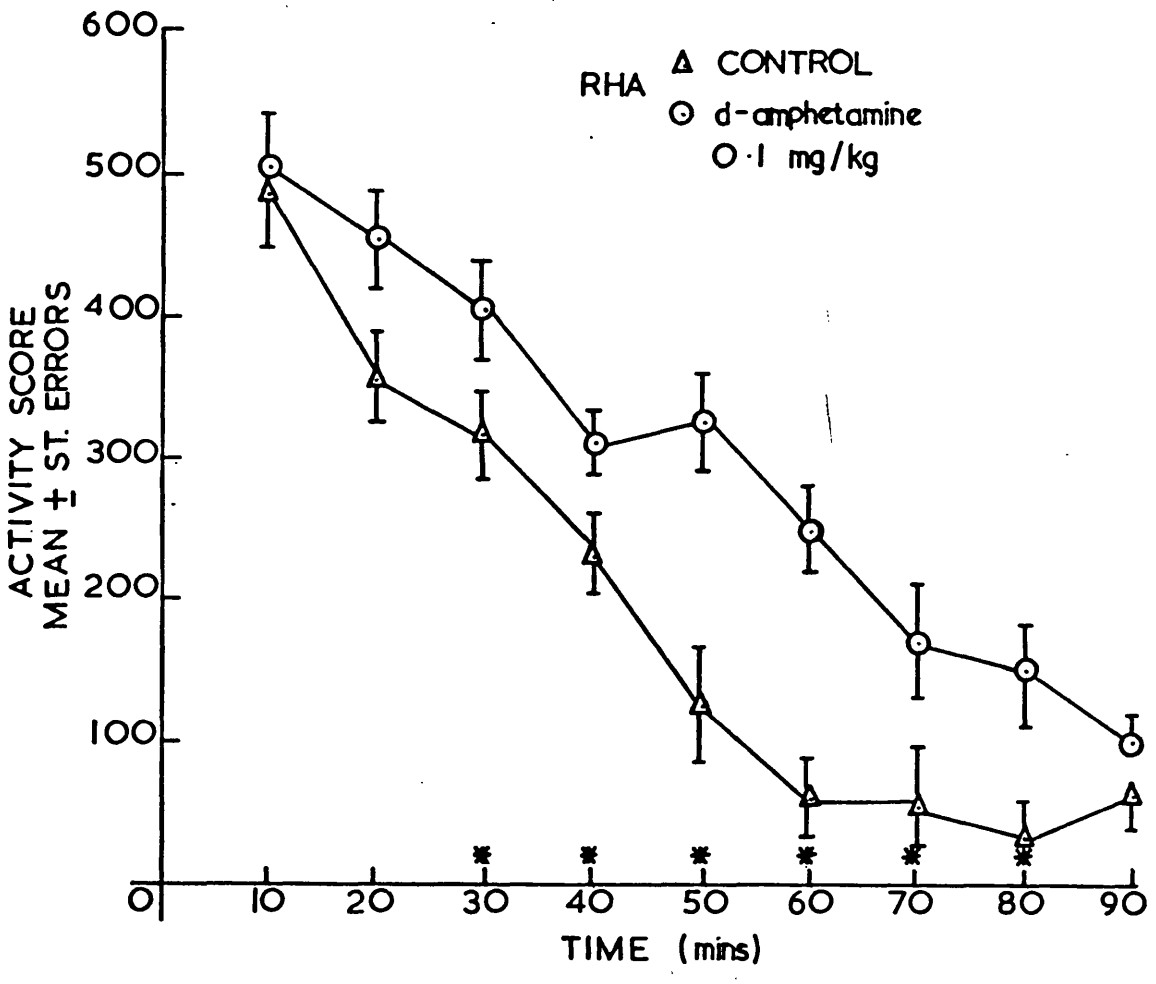


Figure 4-20. Spontaneous activity in RHA strain males injected (s.c.) with d-Amphetamine (0.1 mg/kg.), immediately before placing in recording cages.

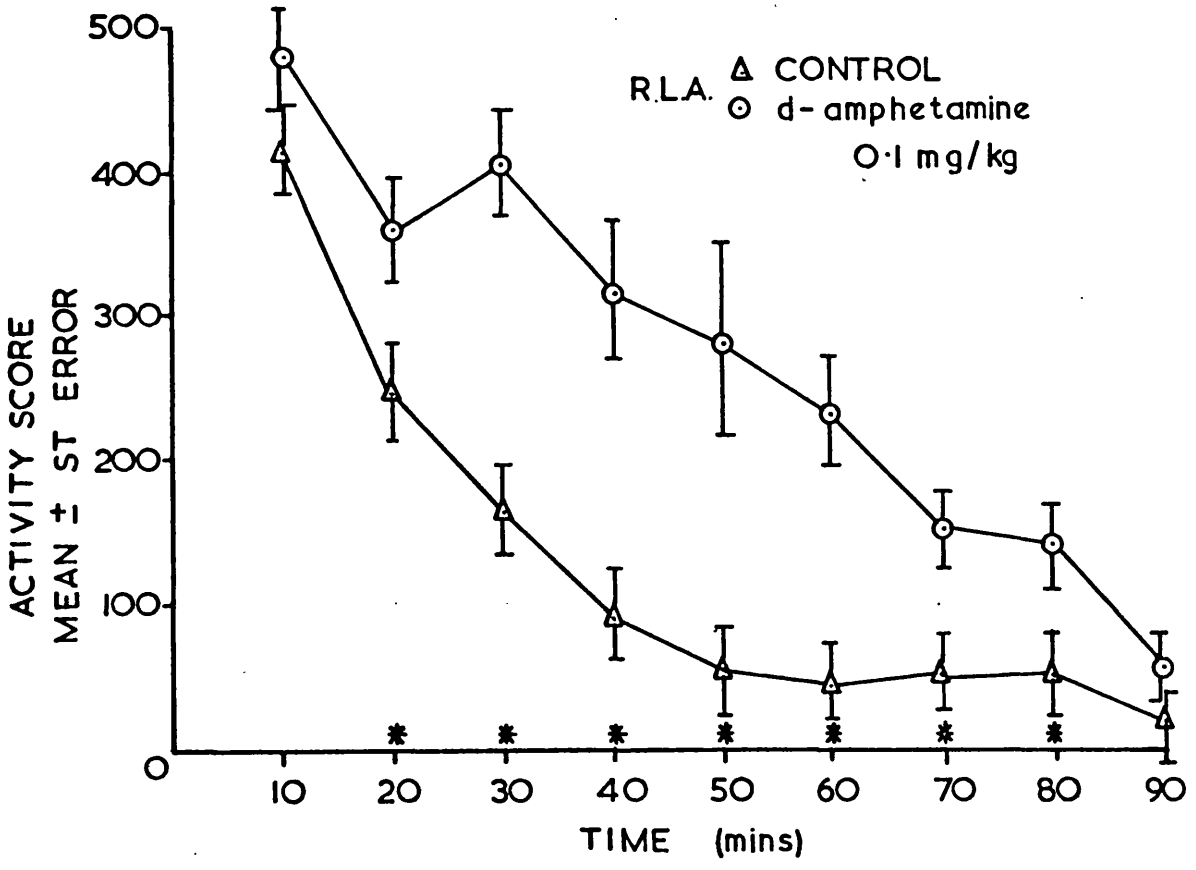


Figure 4-21. Spontaneous activity in RLA strain males injected (s.c.) with d-Amphetamine (0.1 mg/kg.), immediately before placing in recording cages.

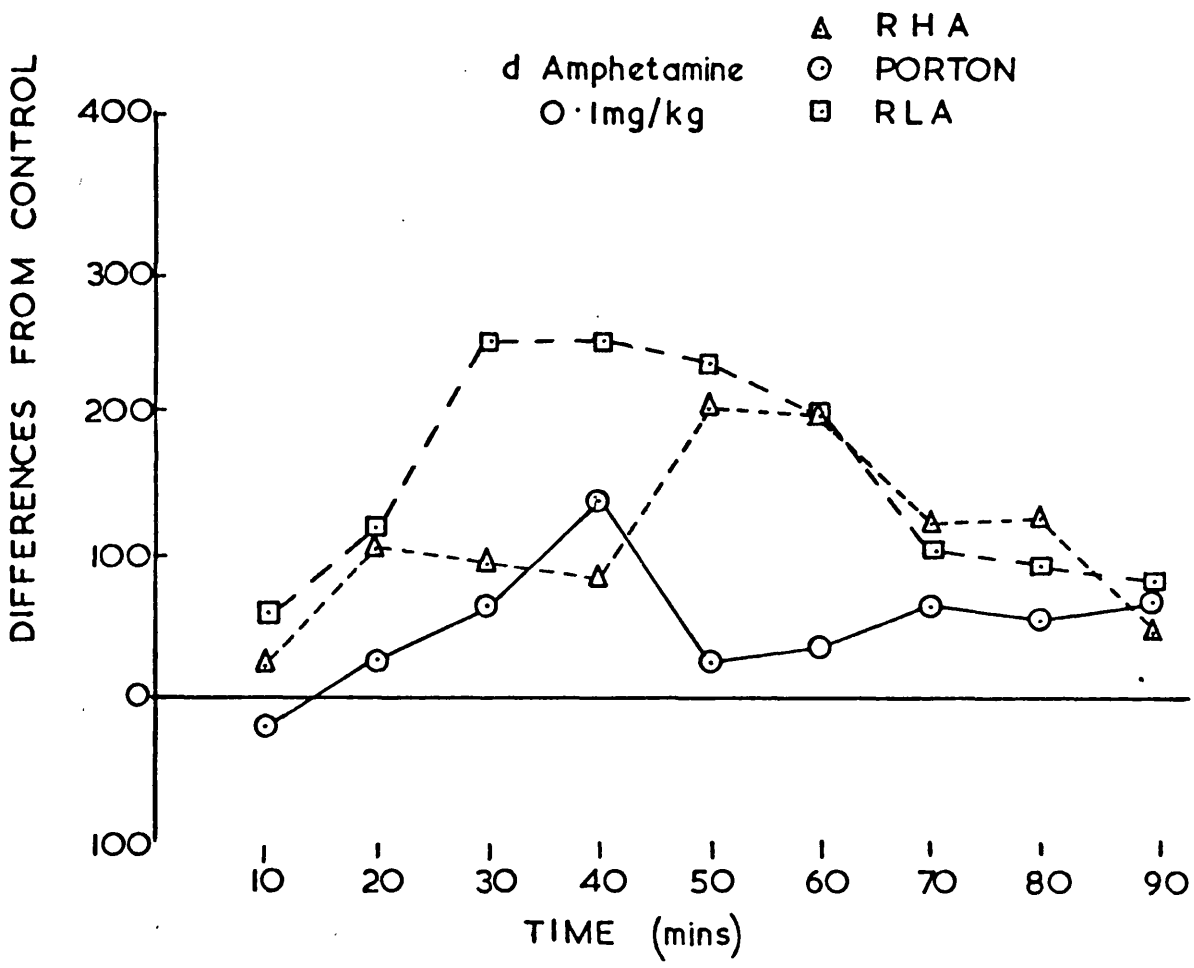


Figure 4-22. Spontaneous activity after d-Amphetamine (0.1 mg/kg.) in the 3 strains. Differences from controls (mean activity scores taken from Figures 4-19, 4-20 and 4-21).

given NEPB, or d-Amphetamine at the same doses, or saline (1.0 ml/kg.). The effects are shown in Figure 4-23. Increased locomotor activity was demonstrated during a short period of the experiment, by the group given the combination of drugs.

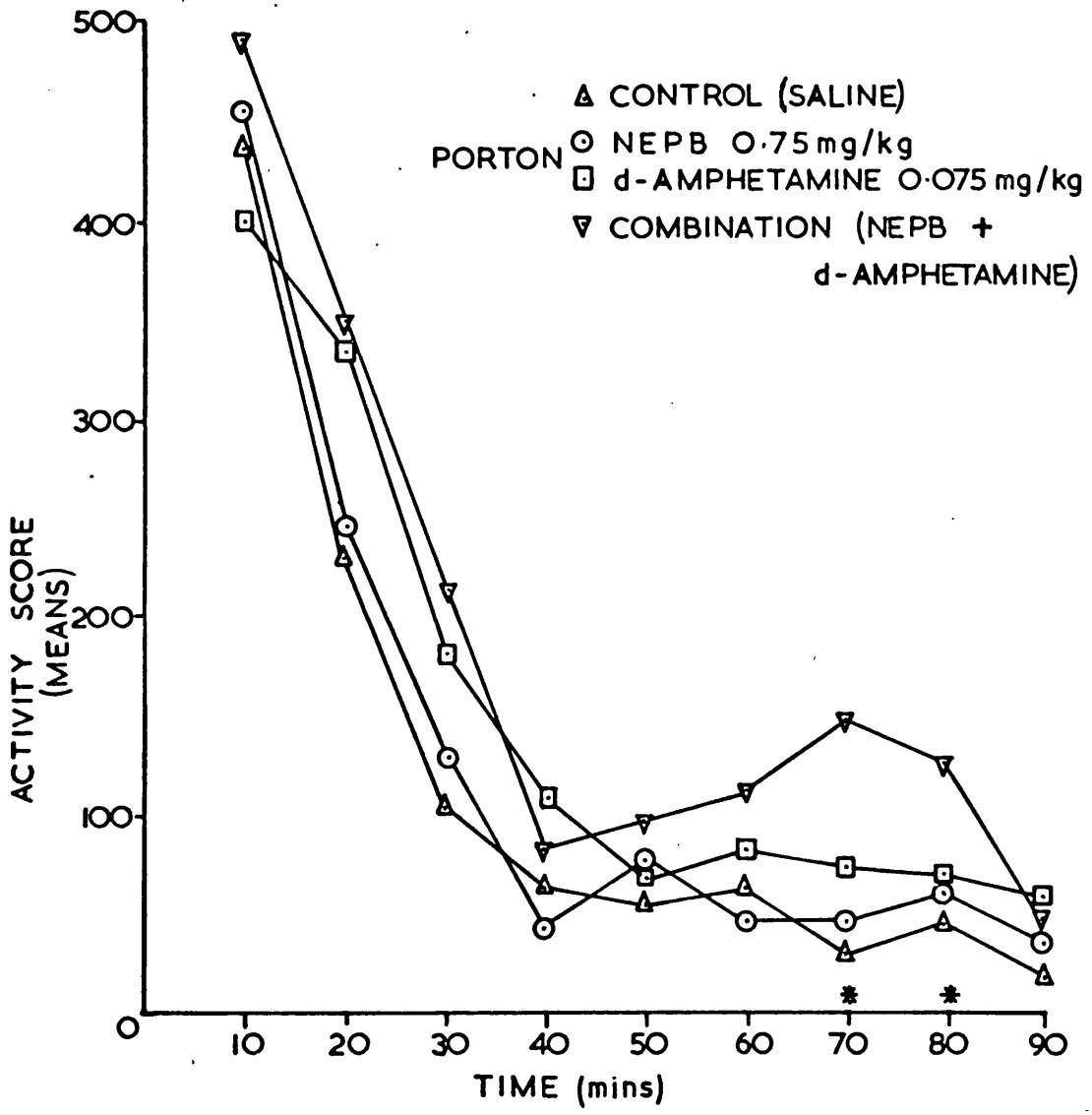


*Figure 4-23. Spontaneous activity of Porton strain rats (10 males per group) after injection with either, NEPB (0.75 mg/kg.) or d-Amphetamine (0.075 mg/kg.) or a combination of both these drugs. Mean activity scores are shown without standard errors. Significance points, shown * , refer to significant differences between the groups receiving the drug combination and the rest of the treatment groups and controls.*

CHAPTER 5

The Extraction and Estimation of Acetylcholine

5.1 Extraction Procedures

Because of the highly labile nature of ACh in fresh nervous tissue it is necessary, to kill the animals and fix the brain tissue rapidly in order to retain the ACh concentration in a form that resembles the in vivo state. No technique available at present can attain this ideal entirely, because: (1) synthesis of new ACh and/or degradation of existing ACh will take place after death,in a way which may not be typical of the living brain. These changes may be very rapid; (2) the occurrence of sudden death may be expected to produce many sudden changes in the state of the brain, amongst these will be massive changes in sensory input, hormonal reactions to sudden stress and sudden reductions in oxygen tension, (3) stress through fear, induced in the animal immediately before death,may render the final living states of the brain 'abnormal'. On the other hand, it is well known that most anaesthetics and hypnotics change the ACh content of the brain (Crossland and Slater, 1968) therefore, it was decided to kill the animals in an unanaesthetised state for this project.

Two techniques for killing the animal and fixing the brain tissue were tried, only one of which was finally used in these experiments.

5.11 Method 1

In order to reduce chemical change in the brain as much as possible,the technique of whole animal freezing was considered as a potentially useful method for this work. The animal was enclosed in a cylindrical wire cage, having a clasp-fastened door at each end and a length of 16 cm and diameter of 4 cm. Rats readily run into the open

door of such a cage and so presumeably do not initially, fear the situation. The animal was then plunged head first into a large, wide-necked flask containing liquid nitrogen, so that at least the anterior half of the body was submerged. Liquid nitrogen was added when necessary to maintain this level and the animal was kept in this position for at least 2 minutes, to ensure complete freezing. On withdrawal from the cage the head was removed from the body with a blow from a chisel and the head clamped to a cooled steel plate for dissection. Using small sharp chisels and scalpels and a chipping technique, the brain was removed from the skull. Some brains were left intact and ACh extracted from whole brain and others were divided into 5 main areas. These areas consisted of the following regions: (1) medulla oblongata and pons; (2) cerebellum; (3) hypothalamus; (5) cortex and (4) the remaining area, which consisted of mid-brain structures and included hippocampus, corpus striatum, amygdala, thalamus, caudate nucleus and other areas. This dissection produced brain areas that were approximately equal to those obtained by method 2 (described later), but because of the difficulty experienced in handling the brittle, frozen tissue it was not always possible to follow a standard procedure for this dissection. During the dissection the tissue was kept frozen by pouring liquid nitrogen onto the brain at intervals. As the portions of brain were removed they were collected and stored temporarily in tubes containing liquid nitrogen, standing in a thermos flask, until the extractions could be performed. The time required for this procedure, from placing the rat in the restraining cage to having frozen brain areas dissected out, was between 10-20 minutes. The extraction technique was common to both methods and is described after method 2.

5.12 Method 2

This method involved the removal and dissection of unfrozen brains and so demanded a quick dissection technique to allow early

fixation of the tissue. The animals were killed by decapitation. As the use of many of the commercially available rat guillotines involve a certain amount of pre-decapitation stress to the subject, during the restraining procedure, the method was judged inappropriate for this work. A manual technique of decapitation was employed, using a pair of heavy scissors. It was possible to decapitate the animal within seconds of lifting it from its home cage and before stress reactions could occur.

Having decapitated the subject the procedure for the extraction was as follows; the roof of the skull was chipped away with small bone forceps and the brain lifted free of the skull with a cooled, round tipped spatula and placed on a glass tile cooled on ice. The brain was complete except for the olfactory lobes which were not included in any of the extractions discussed here. Some extractions were performed on whole brains but in most cases the brain was divided into five regions with a cooled scalpel using a modified version of the brain dissection, described by Glowinski and Iverson (1966) (Figure 5-1). First the cerebellum, medulla oblongata and pons were separated from the rest of the brain with a cut which began vertically and was then angled slightly anteriorly as shown (cut A). The medullary portion (Portion 1) was then separated (cut B) from the cerebellar portion (Portion 2). As pieces became separated they were dropped into labelled, insulated beakers containing liquid nitrogen. The brain was then turned so that the ventral side was exposed and a new cut made through the optic chiasma (cut C), separating an anterior brain portion. A cube of tissue was removed using as limiting edges anteriorly, cut C, and laterally the edges of the cerebal cortex, thus isolating the hypothalamus (Portion 3). The cortical portion of the larger of the remaining brain pieces was then separated from the mid-brain regions using the distinct white layer of the corpus callosum, as a guide. The cortical portion of the remaining anterior brain piece was then separated from the enclosed corpus

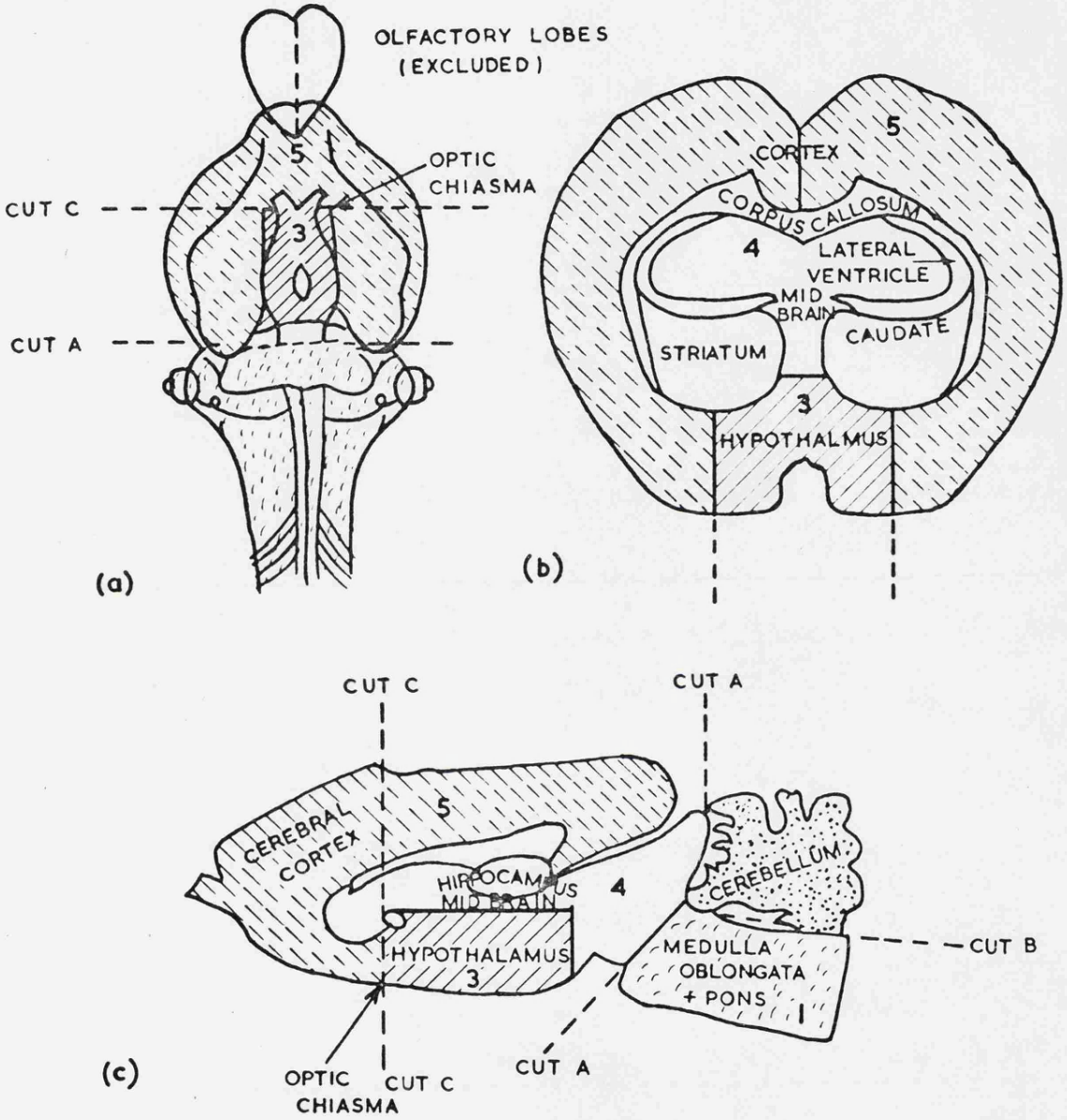


Figure 5-1. Diagrammatic representation showing dissection procedure for the rat brain. (a) Ventral surface, (b) exposed surface after cut C, showing areas (white) included in Area 4 ('mid-brain' sample), (c) lateral view. (Modified after Glowinski and Iverson, 1966).

striatum. The latter was placed with the other 'mid-brain' regions, including the hippocampus, caudate nucleus, thalamus and amygdala (Portion 4) separated from the cortical hemispheres which, together with the anterior cortical piece, formed the cortical sample (Portion 5). All brain portions were weighed on a torsion balance and stored temporarily in tubes containing liquid nitrogen in a thermos flask, prior to ACh extraction. The time taken from decapitation to frozen pieces never exceeded 2 minutes and averaged approximately $1\frac{3}{4}$ minutes.

5.2 Relative usefulness of freezing (Method 1) and non-freezing (Method 2) extraction techniques

5.21 Consistency of brain dissections

When comparing brain regions for ACh concentration it is clearly of paramount importance that identical brain areas should be taken each time. The consistency of the dissections of the five areas was checked by calculating the means and standard deviations of the weights of the different areas obtained by method 1, and by method 2. The area weights were expressed as percentages of total brain weight and are shown in Table 5-1.

	Method 1 (Dissection of frozen brain)	Method 2 (Dissection of unfrozen brain)
1. Cerebellum	14.2 ± 0.90	14.4 ± 0.23
2. Medulla	10.1 ± 1.40	12.0 ± 0.42
3. Hypothalamus	5.2 ± 2.30	4.8 ± 1.50
4. Cortex	53.1 ± 8.30	48.6 ± 0.17
5. Mid-brain	17.4 ± 6.20	20.2 ± 0.23

Table 5-1. Means and standard deviations of brain area weights (% of total brain weight) from 8 brains dissected by methods 1 and 2.

The more consistent weights, as denoted by the small standard deviations, were obtained when the brain was dissected in the unfrozen state. Dissection of frozen brain was less consistent because frozen tissues are very brittle and tend to break unpredictably during dissection. It is also difficult to recognise some brain areas which are obvious in unfrozen tissue and it is even possible to include small pieces of frozen non-nervous tissue (skin, muscle and bone) with brain samples, because of the similar appearance of these tissues when frozen.

Consistency in weight of the dissected brain areas does not necessarily mean that the same areas are being chosen each time, only that the same amount of tissue is taken. However, a lack of consistency by weight, as seen with method 1, shows that even if the correct area is being sampled each time, differing amounts are being taken. Also it is important to note that as the whole brain is divided up, as opposed to isolated areas being selected, to take too much tissue from one zone, means that an adjacent area loses a complimentary amount and the error will be compounded. Thus on the basis of weight consistency, method 2 was superior.

5.22 ACh concentration in whole brains, removed by methods 1 and 2

It was predicted that greater changes in brain ACh concentration would occur in the brains removed by method 2 than in those removed by method 1, which were fixed more rapidly by freezing. In order that a valid comparison could be made, ACh concentration was measured in whole brains removed by both methods. Whole brains were compared to avoid complicating factors due to the weight differences described above for brain areas. The rats used in this comparison were males of the Porton strain and 10 brains were removed by each method. (Assays were performed using the frog rectus abdominis preparation, which is described in Section 5.4).

Total Brain ACh	Method 1	Method 2
µg/gm. of brain	1.85 ± 0.22	1.80 ± 0.32

Comparison of the means by Student's t-test showed a non-significant difference between the two methods. Thus, if changes had occurred in the ACh level of brains removed by method 2, they were compensatory ones, resulting in a final level similar to the original one. Alternatively, changes may have occurred in the rapidly frozen brains which were equivalent to those occurring by method 2.

It was apparent, therefore, that there was nothing to be gained by using the more laborious method 1, for removing the rat brains. In fact, as the brain dissection experiments showed, it was a less than satisfactory method for obtaining specific brain areas for assay. Also large quantities of liquid nitrogen were required and the procedure was more time consuming than method 2. For these reasons, method 2 was preferred for these studies.

5.3 Extraction of ACh

Each brain piece was crushed in a super cooled steel anvil, especially made for this purpose. (Figure 5-2). The frozen brain powder produced was carefully scraped into a glass homogeniser tube bedded in crushed ice and homogenised with cooled 95% acid/ethanol solution (made by adding 0.2 ml. of glacial acetic acid to 100 ml. of 95% ethanol) using 2.0 ml. for each brain portion and 5.0 ml. for whole brains. The brain sample was homogenised for one minute using a teflon pestle (Jencons - Teflon/glass homogeniser) mounted to a stirring motor rotating at a speed of approximately, 1000 r.p.m. The homogenate was collected in a centrifuge tube whilst the homogeniser tube and pestle were washed with

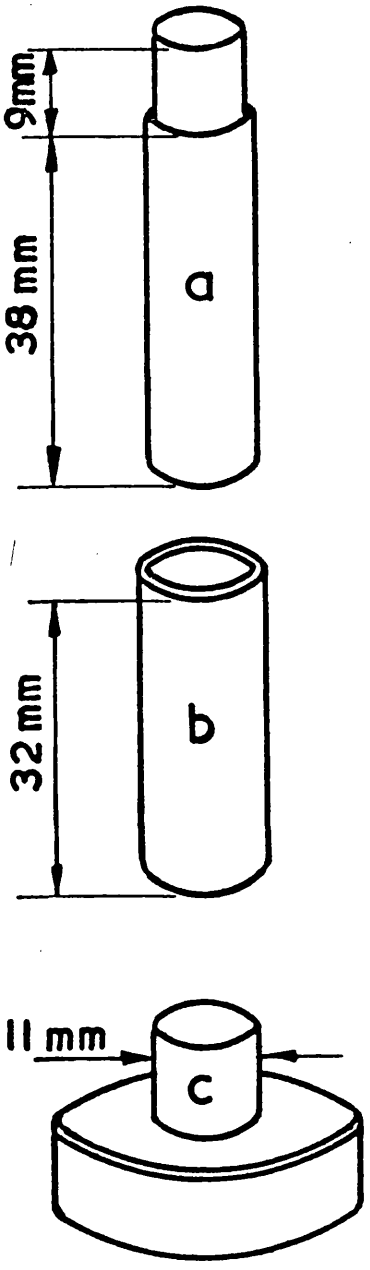


Figure 5-2. Exploded view of stainless steel anvil used to crush frozen brain pieces. Tube (b) is placed over boss (c) and the frozen tissue dropped inside. The plunger (a) is then pushed into the tube and given a sharp blow with a hammer. The frozen tissue is compacted into a plug of powder which adheres to the tube from which it can be scraped into the homogeniser tube. The dimensions given describe the anvil used for brain areas. A slightly larger anvil was used for crushing whole brains.

a further volume of 95% acid/ethanol solution (1.0 ml. for brain portions or 2.0 ml. for whole brains). This wash was also added to the original homogenate. Samples were allowed to stand for a minimum of 20 minutes on ice before centrifugation for 10 minutes at approximately, 420 g. The supernatants were collected in glass tubes (Quickfit) standing in ice whilst the brain residue was washed with 2.0 ml. of 75% acid/ethanol (made by adding 0.15 ml. of glacial acetic acid to 100 ml. of 75% ethanol) by shaking the residue with the washing solution and centrifuging for a further 10 minutes at approximately, 420 g. This wash was added to the supernatant. 1.0 ml. of distilled water was then added to each tube to precipitate proteins in the extract and the volume reduced to approximately, 1.0 ml. in a rotary 'Evapo-mix' (Buchler Instruments) at 37°C. Finally, the extracts were adjusted to pH 4.0 and stored below 4°C until assayed.

5.4 Biological assay of ACh

The quantitative assessment of ACh is still very much dependant upon longstanding techniques of biological estimation. A fluorometric technique of assessment has been reported (Fellman, 1969), but problems due to unreliability still exist. Some workers, for example, have reported an inability to repeat the work of Fellman (Hunt 1972, Redfern, personal communication 1972). However, Jenden, Campbell and Roch (1970) have described a gas chromatographic technique for estimating ACh in tissues, the usefulness of which has been confirmed by Metcalfe (personal communication, 1973). Recently, Goldberg and McCaman (1973) have described an enzymatic radioassay technique which has great sensitivity and may permit estimation of very small quantities of tissue ACh. Several biological estimation techniques are available and have shown reliability over a number of years. It was decided to use biological methods in this work and two different methods were used.

5.41 *Frog rectus abdominis muscle preparation*

This is a commonly used preparation for the estimation of ACh and is fully described by Richter and Crossland (1949). The method was chosen because of its adequate sensitivity, after physostigmine treatment, for this type of work and because the preparation has a robustness and longevity not seen with some alternative preparations, such as the guinea-pig ileum preparation.

The muscle preparations were obtained from Rana spp. which were kept in a partially covered tank containing some fresh water. The frogs were stunned, decapitated and the spinal cord destroyed. The rectus abdominis muscle was removed whole, if the frog was small, or divided into 2 pieces if large and transferred to a dish containing frog-Ringer solution. (Bunzle's Ringer, containing; NaCl 111.0 mM; $CaCl_2$ 1.09 mM; KCl 1.87 mM; Phosphate Buffer (30 ml./litre of solution made by adding NaH_2PO_4 (147.4 mM) to Na_2HPO_4 (148.0 mM) to give pH 7.4); Glucose 11.11 mM.) Lengths of thread were sewn through and tied, to each end of the muscle which was then suspended in a 4 ml. organ-bath containing frog-Ringer solution. The top thread being attached to an aluminium lever and the bottom thread anchored to a wire loop in the base of the organ-bath. A small electric pump aerated the bath which was maintained at room temperature (Figure 5-3).

An optical-wedge transducer (Devices Ltd.) was used to detect changes in muscle length and changes in electrical potential produced were recorded, via a matched amplifier, on a galvanometric pen-recorder (Evershed and Vignoles).

Before starting the assays, a weight (0.5 - 1.0 gm, Plasticine) was suspended from the lever on the optical-wedge side of the pivot to tension the muscle which was allowed to stretch in this way for 45 minutes in frog-Ringer, followed by a further 45 minutes in frog-Ringer containing Physostigmine salicylate (10 µg/ml.).

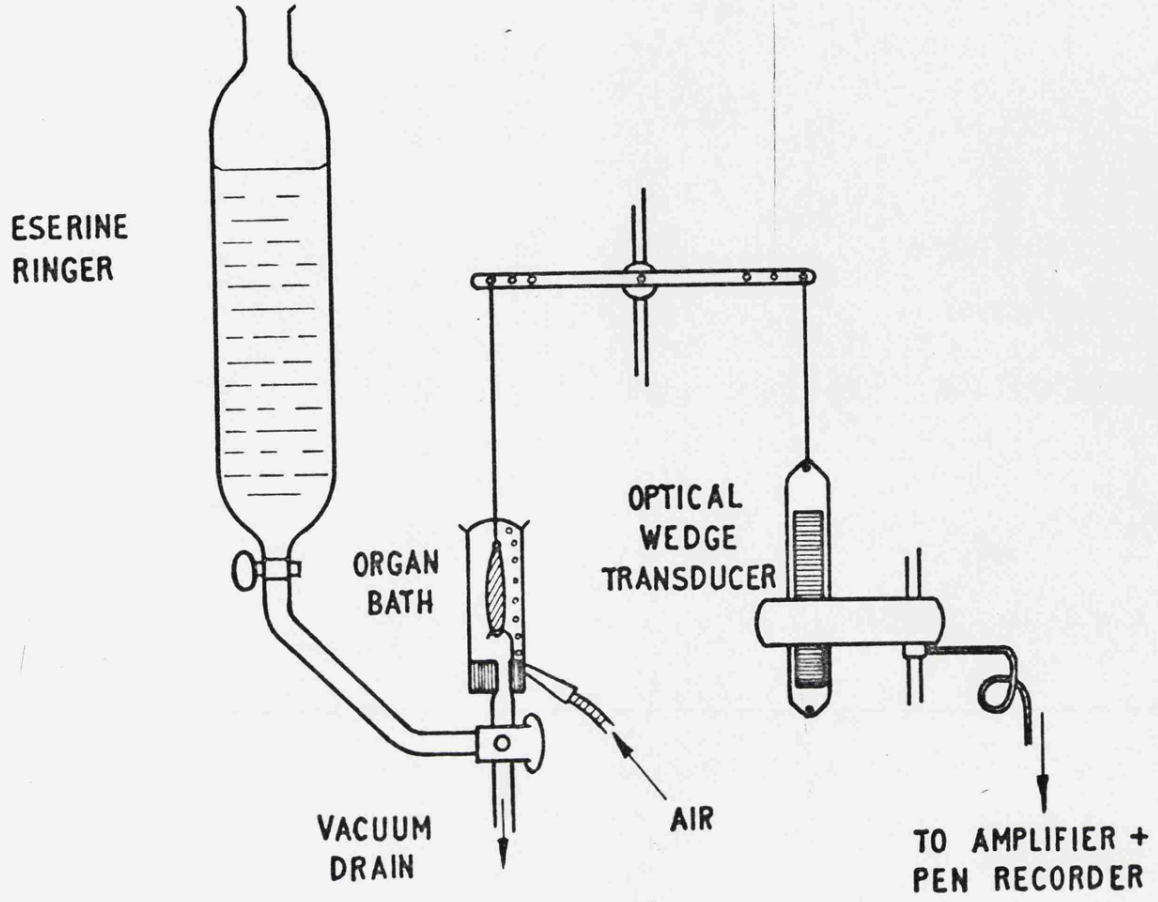


Figure 5-3. Arrangement of bio-assay equipment for frog and leech muscle assays. For the leech preparation an organ bath of 1.0 ml. capacity was used which was filled via the bottom of the bath from the reservoir of Ringer solution. A 4.0 ml. capacity bath was used for the frog muscle and a stretching weight of 0.5 - 1.0 gm. was added to the right-hand side of the lever between contractions. Ringer solution was added to the frog muscle from the top of the bath with a pipette.

Because it was difficult to accurately fill the small organ-bath to the same level each time, drugs and extracts of brain were added to the muscle in the bath by 'complete change method', i.e. the ACh or extract was made up in frog-Ringer and used to replace the bathing fluid instead of injecting small quantities of drug into the bath. The contraction cycle used was as follows:

0 s: Remove weight from lever and start pen-recorder.

30 s: Drain organ-bath, add drug solution in frog-Ringer and start clock.

120 s: Switch off pen-recorder, drain organ-bath and fill with frog-Ringer, drain again and fill with frog-Ringer plus physostigmine. Replace weight on lever to stretch muscle to original length.

300 s: Remove weight for a new cycle.

5.42 *Leech dorsal muscle-micropreparation*

The frog-rectus abdominis preparation was found to be sensitive enough for all but certain low ACh concentration areas for which a modified version of the leech dorsal muscle preparation was employed. Leech preparations are generally found to have greater sensitivity than frog rectus abdominis (Pharmacological Experiments on Isolated Preparations, Publ. Livingstone 1970). A modification of the 'whole muscle' preparation was used (Southward and Kerkut, personal communication 1972) which employed a small piece of dorsal muscle.

Leeches (Hirudo medicinalis) were stored in a large glass beaker containing distilled water, at room temperature. To dissect; a leech was pinned out in an extended position in a wax-lined dish and a longitudinal incision made the length of the body. After removal of the internal organs, an area of the muscle wall was cleared of botryoidal tissue and a strip of muscle delineated by two parallel cuts, approxi-

mately 3 mm apart and 10 mm long. Threads were tied around the ends of the strip before cutting it free of the body wall and mounting it in a 1.0 ml. glass organ-bath containing leech-Ringer solution, (Leech-Ringer solution containing; NaCl 115 mM, $CaCl_2$ 2 mM, KCl 4 mM, Tris-Buffer 10 mM and glucose 10 mM), with physostigmine salicylate (10 μg/ml.) and aerated with an electric pump. This preparation is very sensitive to mis-handling so great care was taken during dissection and setting up to avoid unnecessary contact with dissection instruments and to avoid undue stretching of the preparation. The muscle was arranged for recording purposes, as for the frog muscle preparation except that a smaller lever was employed and no stretching weight was used (Figure 5-3). The muscle was allowed 1 hour to settle down before use. To reduce physical disturbance to the muscle, drugs and extract solutions were added to the bath from a micro-syringe (Shandon-Terumo) and the bath was only drained at the end of a contraction period and the subsequent wash. Leech-Ringer was added from the base of the bath by opening a tap in a tube to a Ringer header resevoir. The organ-bath was constructed so that filling it to the top (top of meniscus level with lip of bath) required fractionally less than 1.0 ml. of Ringer. Adding drug solutions brought the volume very close to 1.0 ml. The contraction cycle used was as follows:

0 min: Start recorder, add drug to organ-bath and start clock.

2 min: Stop recorder, drain organ-bath, refill with fresh Ringer, drain again and refill.

22 min: Start recorder and begin new cycle.

Some leech preparations required up to 30 minutes to relax fully.

A simple 'bracket assay' technique was used for frog and leech preparation assays with a minimum of four contractions to an assay,

i.e. at least two responses to a standard ACh solution and two to the brain extract were used.

The brain extracts were prepared for both types of assay as follows: the stored extract was made up to 4 ml. with distilled water and divided accurately into 2 equal portions. The test portion was adjusted to pH 7.0 by adding dilute caustic soda, and made up to 4 ml. with frog or leech-Ringer, according to the preparation to be used. The remaining portion was used to control for ACh potentiating substances that may have been present in the extract according to the method recommended by Feldberg (1945). This portion was adjusted to pH 11.0 and boiled for 45 s. When cool it was adjusted to pH 7.0 and made up to 4 ml. with the appropriate Ringer solution. An equal volume of this solution as was employed to give a contraction with the test portion of extract, was added to all doses of standard ACh solution used.

5.43 The Relative usefulness of Frog and Leech preparations

Used in the way described the frog rectus abdominis preparation usually showed a sensitivity which allowed the measurement of ACh at concentrations down to 12.5 nanograms/ml. in the organ-bath, whilst some leech muscle preparations gave measurable contractions in response to ACh concentrations down to approximately 5.0 nanograms/ml.

Although the frog muscle preparation was less sensitive than the leech it was consistently an easier preparation to set up and use. Nearly all frog preparations could be expected to have a useful sensitivity level and frequently a preparation could be used for 2 consecutive days. The leech preparation, however, was of limited use because of its tendency to change sensitivity with time, and the difficulty experienced in relaxing the muscle between contractions. For these reasons, leech muscle preparations were only used for assay of certain brain areas, which it was predicted, contained very low ACh concentrations. The validity of

comparing ACh estimations obtained with different assay methods was checked by dividing a few brain extracts equally and assaying the halves with the different techniques. No significant differences were demonstrated between techniques.

5.5 Brain ACh levels in normal rats

5.51 Comparison of Brain ACh levels in the Three Strains (Whole Brain)

Rats of the Porton, Roman High Avoidance (RHA) and Roman Low Avoidance (RLA) strains taken at approximately 100 days of age, were killed and the brains removed by method 2 described above. Extracts were made from whole brains and were assayed using the frog rectus abdominis preparation and the ACh content expressed in µg/gm of brain tissue (means ± standard deviations) (Table 5-2). It can be seen from the table that the RLA strain demonstrated a significantly higher level of ACh in the whole brain than the RHA strain, whilst the Porton strain possessed an intermediate level of ACh which was not significantly different from that of either of the Roman strains.

5.52 Comparison of ACh levels in Discrete Areas of the Brain

Male rats of the Porton, RHA and RLA strains were taken at approximately 100 days of age and killed and the brains removed according to method 2. This time the brains were dissected into brain regions and the extracts assayed for ACh content. Frog rectus abdominis muscle was used for all areas except some of the cerebellar portions which were assayed using the leech muscle micropreparation. The brains from 8 rats of each strain were used.

The ACh content, expressed in µg/gm (means ± standard deviations), is shown in Table 5-3. First it can be seen that the area of highest ACh concentration in all the strains, was the hypothalamus and the second highest the mid-brain region. The medulla and cortex contained approximately equal quantities in the next highest concentration, whilst the

Strain	No. of Brains	Whole Brain ACh (μg/gm)	Significance (Probability)
Porton	20	1.88 ± 0.42	
RHA	19	1.73 ± 0.34	P > 0.01
RLA	18	2.06 ± 0.34	

Table 5-2. ACh concentration in whole brain of Porton, RHA and RLA rats (males). The statistical probability is based on a comparison of the mean values by Student's t-test. The Porton strain was not significantly different from either of the Roman strains.

Brain Area	Porton	RHA	RLA	Significance (P) Porton RHA	Porton RLA	RHA RLA
1	2.14 ± 1.48	1.01 ± 0.64	2.96 ± 1.55	NS	NS	P>0.002
2	0.98 ± 0.45	0.80 ± 0.12	1.15 ± 0.10	NS	NS	P>0.001
3	4.86 ± 1.45	5.16 ± 2.90	7.96 ± 2.55	NS	P>0.01	P>0.05
4	3.31 ± 1.65	3.51 ± 0.89	4.55 ± 2.74	NS	NS	NS
5	1.96 ± 0.80	2.06 ± 0.37	2.61 ± 0.43	NS	P>0.001	P>0.02

Table 5-3. ACh concentration in brain areas of Porton, RHA and RLA rats (males). Brain areas: 1, medulla and pons; 2, cerebellum; 3, hypothalamus; 4, mid-brain; 5, cortex. Probability values based on comparison of means using Student's t-test. NS, not significant.

cerebellum contains the least of all. The between strains comparison shows that the greatest differences are demonstrated between the RHA and RLA strains. In each area, except area 4, the RLA strain had a significantly higher concentration of ACh than the RHA strain. The Porton strain had significantly less ACh than the RLA strain in two brain areas but did not differ significantly from the RHA strain in any area.

5.6 The Effects of Drugs on Brain ACh levels in the 3 strains

5.61 The Effects of Anti-cholinesterase Drugs

Two anti-cholinesterase drugs, Physostigmine salicylate and Pyridostigmine hydrobromide were examined for their effects on the levels of ACh in brain areas of the three strains. The doses and strains used were as follows; Porton strain; physostigmine 0.5, 0.25 and 0.125 mg/kg. and pyridostigmine 0.25 mg/kg.; RHA and RLA strains; physostigmine 0.25 and 0.125 mg/kg. and pyridostigmine 0.25 mg/kg. The two lowest doses corresponded to doses of these drugs employed in the behavioural experiments described in previous chapters. Drugs were dissolved in physiological saline and injected, subcutaneously, (s.c.) 30 minutes before sacrificing the animals; this timing coincides with the time of approximate peak effect on spontaneous activity of physostigmine in experiments described above. After injection of a drug the animals were placed in a carrying box where they remained undisturbed until sacrificed. The extracts were assayed as above using frog rectus abdominis and leech muscle preparations.

The results of the physostigmine and pyridostigmine experiments are shown in Tables 5-4 (Porton), 5-5 (RHA) and 5-6 (RLA). The effect of physostigmine was to increase levels of ACh in most brain areas of the three strains and the effects were also dose dependent. A high dose (0.5 mg/kg.) of physostigmine was given to the Porton strain rats as a

pilot experiment to discover if effects on ACh level were detectable. ACh levels were significantly increased in several brain areas by this dose and it was decided to use smaller doses in further experiments with all strains. It was therefore possible to use the same doses of physostigmine for the behavioural experiments and ACh estimation studies.

The tables of results show the mean ACh concentrations (μg/gm brain, wet weight) for brain areas. The ACh concentrations, after drug treatment, were compared to the control values for that brain area (i.e., the same values as shown in Tables 5-2 and 5-3). The percentage change in level is shown and Student's t-test was employed to compare each drug treated brain level with the control level for significant differences. It should be noted that the percentage changes shown are a guide to the extent of the change only, as variation in some groups is relatively high and therefore a percentage expression may, occasionally be misleading. For this reason statistically significantly differences are discussed rather than percentage differences.

Strain differences were observed and also the degree to which different brain areas were affected by physostigmine. The medulla was consistently affected, showing significantly increased ACh concentration in all the strains and at all doses used except one. (Table 5-6, RLA strain, 0.125 mg/kg.). On the other hand, the hypothalamus, an area of high concentration ACh, only once showed a significant change after physostigmine (Table 5-5, RHA strain, 0.125 mg/kg.). The 'mid-brain' portion and the cortex were also frequently affected to a significant degree but no particular area was affected in a fashion which was consistently related to strain or dose. The RHA strain showed the greatest sensitivity to AChE inhibition in terms of increased ACh.

Although relatively high percentage increases in medullary ACh were seen after pyridostigmine treatment of the RHA and Porton strains, neither these nor any other brain areas of the three strains showed significant changes in ACh concentration.

Brain area	Control	Physostigmine 0.5 mg/kg.	% change	Physostigmine 0.25 mg/kg.	% change	Physostigmine 0.125 mg/kg.	% change	Pyridostigmine 0.25 mg/kg.	% change
1	2.14 ± 1.48	4.12 ± 0.94*	+93	5.76 ± 1.63**	+169	2.77 ± 0.80*	+29	3.10 ± 1.50	+45
2	0.98 ± 0.45	1.39 ± 0.90	+42	1.21 ± 0.85	+ 23	1.30 ± 0.38	+33	1.10 ± 0.61	+12
3	4.86 ± 1.45	4.68 ± 1.77	- 4	4.18 ± 0.38	-13	4.21 ± 1.45	-13	4.80 ± 1.83	-12
4	3.31 ± 1.65	5.75 ± 0.87**	+74	6.19 ± 1.18**	+87	3.93 ± 0.45	+19	3.17 ± 1.45	- 4
5	1.96 ± 0.80	3.91 ± 0.90**	+99	3.01 ± 0.45**	+53	2.03 ± 0.34	+ 4	1.99 ± 0.78	- 2

*Table 5-4. ACh concentration in discrete brain areas of the Porton rat (6 males/group) after injecting Physostigmine or Pyridostigmine, (s.c.) 30 minutes before sacrifice. ACh concentration expressed in µg/gm of brain tissue (mean ± standard deviation). Significance levels (compared to controls): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.*

Brain area	Control	Physostigmine 0.25 mg/kg.	% change	Physostigmine 0.125 mg/kg.	% change	Pyridostigmine 0.25 mg/kg.	% change
1	1.01 ± 0.64	4.70 ± 2.70*	+365	5.19 ± 1.21***	+413	1.81 ± 1.63	+79
2	0.80 ± 0.12	1.21 ± 0.14***	+ 51	2.11 ± 0.91*	+163	0.84 ± 0.24	+ 5
3	5.16 ± 2.9	6.70 ± 1.82	+ 30	11.75 ± 6.8*	+127	3.70 ± 0.84	-28
4	3.51 ± 0.89	4.68 ± 1.30	+ 33	5.95 ± 2.40	+ 70	2.39 ± 1.34	-32
5	2.06 ± 0.37	3.31 ± 0.84*	+ 61	2.77 ± 0.78	+ 34	1.73 ± 0.32	-16

Table 5-5. ACh concentration in discrete brain areas of RHA rats (6 males/group) after injecting Physostigmine and Pyridostigmine; (s.c.), 30 minutes before sacrifice. ACh concentration expressed in µg/gm of brain tissue (mean ± standard deviation). Significance levels as for Table 5-4.

Brain area	Control	Physostigmine 0.25 mg/kg.	% change	Physostigmine 0.125 mg/kg.	% change	Pyridostigmine 0.25 mg/kg.	% change
1	2.96 ± 1.55	7.22 ± 1.61	+144	3.80 ± 0.84	+28	2.74 ± 1.21	-7
2	1.15 ± 0.10	1.52 ± 0.14**	+ 32	1.21 ± 0.20	+ 5	1.08 ± 0.15	-6
3	7.96 ± 2.55	8.41 ± 1.20	+ 6	6.83 ± 3.00	-14	7.52 ± 1.82	-6
4	4.55 ± 1.74	7.03 ± 1.23*	+ 54	5.28 ± 1.02	+16	4.61 ± 1.82	+1
5	2.61 ± 0.43	3.59 ± 0.22*	+ 38	3.41 ± 1.01*	+33	2.80 ± 0.45	+7

Table 5-6. ACh concentration in discrete brain areas of RLA rats (6 males/group) after injecting Physostigmine and Pyridostigmine, (s.c.), 30 minutes before sacrifice. ACh concentration expressed in μg/gm of brain tissue (mean ± standard deviation). Significance levels as for Table 5-4.

5.62 The Effects of Anti-Aceytylcholine Drugs and d-Amphetamine

The results of the experiments in which the anti-ACh drugs, NEPB and NEPB MeI, and also d-Amphetamine were given are shown in Tables 5-7 (Porton), 5-8 (RHA), and 5-9 (RLA). Significant reductions in ACh concentration were observed in all the strains after NEPB but only in certain areas. The percentage changes were always relatively smaller than those measured after physostigmine and also there was a tendency for different areas to show changes. Thus the medulla was not affected at this dose by NEPB but was consistently changed after physostigmine. The cortex was the area most affected in these experiments with the RLA strain experiencing a reduction in ACh of the greatest significance.

The quaternary analogue of NEPB did not produce any significant changes in ACh levels and nor did d-Amphetamine.

Brain area	Control	NEPB 1.0 mg/kg.	% change	NEPB MeI 1.0 mg/kg.	% change	d-Amphetamine 0.5 mg/kg.	% change
1	2.14 ± 1.48	2.04 ± 0.64	- 5	2.24 ± 0.84	+ 5	2.76 ± 0.72	+29
2	0.98 ± 0.45	0.81 ± 0.45	-17	0.78 ± 0.55	-20	1.01 ± 0.42	+ 3
3	4.86 ± 1.45	2.82 ± 1.38***	-42	3.94 ± 1.22	-17	3.89 ± 0.68	-20
4	3.31 ± 1.65	2.14 ± 0.78	-35	3.24 ± 0.54	- 2	3.01 ± 0.54	- 9
5	1.96 ± 0.80	0.88 ± 0.10*	-55	2.04 ± 0.84	+ 4	1.97 ± 0.10	+ 1

Table 5-7. ACh concentration in discrete brain areas of the Porton strain rat (6 males/group) after NEPB, NEPB MeI and d-Amphetamine, injected 30 minutes before sacrifice. ACh concentration expressed in μg/gm of brain tissue (mean ± standard deviation) Significance levels as for Table 5-4.

Brain area	Control	NEPB 1.0 mg/kg.	% change	NEPB MeI 1.0 mg/kg.	% change	d-Amphetamine 0.5 mg/kg.	% change
1	1.01 ± 0.64	0.88 ± 0.45	-13	1.14 ± 0.54	+13	1.42 ± 0.89	+41
2	0.80 ± 0.12	0.78 ± 0.34	- 3	1.10 ± 0.42	+38	0.91 ± 0.42	+14
3	5.16 ± 2.9	3.02 ± 1.42	-42	4.72 ± 1.40	- 9	4.99 ± 1.20	- 3
4	3.51 ± 0.89	1.90 ± 0.82*	-46	3.17 ± 0.94	-10	3.32 ± 0.74	- 5
5	2.06 ± 0.37	1.32 ± 0.42**	-36	2.48 ± 1.21	+20	2.15 ± 0.43	+ 4

Table 5-8. ACh concentration in discrete brain areas of the RHA strain rat (6 males/group) after NEPB, NEPB MeI and d-Amphetamine, injected 30 minutes before sacrifice. ACh concentration expressed in µg/gm of brain tissue (mean ± standard deviation). Significance levels, as for Table 5-4.

Brain area	Control	NEPB 1.0 mg/kg.	% change	NEPB MeI 1.0 mg/kg.	% change	d-Amphetamine 0.5 mg/kg.	% change
1	2.96 ± 1.55	2.37 ± 0.83	-20	2.45 ± 1.22	-17	2.52 ± 1.41	-30
2	1.15 ± 0.10	1.01 ± 0.14	-12	1.20 ± 0.43	+ 4	1.19 ± 0.22	+ 4
3	7.96 ± 2.55	5.14 ± 1.22	-37	7.04 ± 2.14	-11	8.01 ± 2.31	+ 1
4	4.55 ± 1.74	2.30 ± 1.14*	-49	3.96 ± 2.11	-12	4.32 ± 1.52	- 5
5	2.61 ± 0.43	1.33 ± 0.43***	-49	2.64 ± 0.82	+ 1	2.72 ± 0.56	+ 4

Table 5-9. ACh concentration in discrete brain areas of the RLA strain rat (6 males/group) after NEPB, NEPB MeI and d-Amphetamine, injected 30 minutes before sacrifice. ACh concentration expressed in μg/gm of brain tissue (mean ± standard deviation). Significance levels as for Table 5-4.

CHAPTER 6

Determination of Brain Acetylcholinesterase Activity

Determinations of AChE activity were made of whole brains and also of discrete brain areas from undrugged rats of the three strains, using the method of Ellman, Courtney, Andres and Featherstone (1961). The method involved the substitution of acetylthiocoline for ACh as substrate for the enzyme. Thiocholine produced during hydrolysis reacts with a colour reagent, dithiobisnitrobenzoate ion (DTNB), by following the increase in yellow colour produced, the enzyme activity may be measured. The method is based on the coupling reactions:

$$\text{acetylthiocholine} \xrightarrow{AChE} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + DTNB \longrightarrow \text{yellow colour}$$

The reaction between thiocholine and DTNB has been shown to be sufficiently rapid so as not to be rate limiting and in the concentrations of substrate used does not limit the enzyme hydrolysis. The rate of colour production was measured on a Pye Unicam SP500 spectrophotometer.

Procedure

Unanaesthetised rats were sacrificed and the brains removed and dissected using the method described for the estimations of brain ACh (i.e., method 2, described in full in Chapter 5). Five rats were used from each strain. Brain areas were weighed and homogenised in phosphate buffer (pH 8.0; 0.1 M) at approximately 2000 r.p.m., using a teflon pestle (Jencons) for 1.0 minute, then diluted to give a final concentration of 1.0 mg/ml. wet weight. Homogenates were stored in a deep freeze until estimated, at which time, homogenates from the three strains were estimated together using the same reagent solutions.

Reagents used:

1. Colour reagent: DTNB (39.6 mg) and sodium bicarbonate (15.0 mg) dissolved in 10.0 ml. of 0.1 M phosphate buffer (pH 8.0) giving a final concentration of 0.1 M.

2. Substrate: Acetylthiocholine iodide (21.67 mg/ml.) 0.75 M dissolved in distilled water.

In a typical run the cuvette volume of 3.0 ml. consisted of brain homogenate, 1.0 ml. (1.0 mg/ml.); DTNB solution, 1.0 ml. (diluted 1:40 with distilled water) and acetylthiocholine, 1.0 ml. giving a final cuvette concentration of 0.5 mM. This was incubated for 1.0 minute 37°C. The solution was then transferred to the spectrophotometer and the change in optical density was measured for 6.0 minutes at 412 mμ. The results were expressed as μm of acetylthiocholine hydrolysed/min/gm protein (1.0 ml. (1.0 mg/ml) of the brain homogenate was used to determine the protein content using the method of Lowry, Rosebrough, Farr and Randall 1951).

The kinetic properties of the enzyme were studied by calculating the K_m and V_{max} values on whole brain homogenates for each strain. The V_{max} is the maximum velocity of hydrolysis of substrate (expressed as μm/min/gm protein) and an expression of the number of receptors available on the enzyme for the substrate. The K_m (Michaelis constant) is the substrate concentration required to yield half the maximum velocity and is a measure of the affinity of the substrate for the enzyme.

The kinetic properties of the ChE in whole brain homogenates of the three strains was studied to determine the optimal substrate concentration for each strain to be used later in the comparison of brain areas in the three strains.

The K_m and V_{max} values were determined by using substrate concentrations from 0.01 to 10.0 mM. An acetylthiocholine blank was esti-

mated and subtracted from the enzyme in optical density produced by the tissue enzyme, at each substrate concentration. The K_m was estimated by using the Lineweaver-Burke plot and the values checked using a computer programme (Berry, 1972) which determined V_{max} and K_m values.

The following values were determined for whole brains:

	K_m values	V_{max} values
Porton	0.90×10^{-4} M	0.74×10^{-3} M
RHA	0.47×10^{-4} M	0.66×10^{-3} M
RLA	0.57×10^{-4} M	0.78×10^{-3} M

No significant differences were noted between the three strains in K_m and V_{max} values, and an optimal substrate concentration of 0.5×10^{-3} M was chosen to determine the enzyme activity in the 5 brain areas.

The range of K_m values for whole brains of the three strains ($0.47 - 0.9 \times 10^{-4}$ M) is within the normal range of biological variation. This indicates that there is no difference in the affinity of the ACh for the AChE receptor. The V_{max} values were also within the limits of normal biological variation and therefore the optimum substrate concentration chosen could be expected to demonstrate any difference in the levels of AChE in the 5 brain areas. The AChE activities determined for the brain areas of the three strains are shown in Table 6-1.

The relative activities of AChE found in the brain areas agree well with those reported by Bennett, Diamond, Morimoto and Herbert (1966) although within group variation is high in the present determinations. Inbred strains of rat are normally recommended for such work when variation can be reduced to approximately 5%, but in this work the randomly bred strains used showed variation in AChE activities, which was sometimes in excess of 10%

Brain Area	Porton	RHA	RLA
1	80.4 ± 8.8	82.3 ± 10.6	84.5 ± 13.4
2	47.3 ± 9.8	46.7 ± 4.3	41.9 ± 3.6
3	156.8 ± 16.4	156.1 ± 14.9	148.7 ± 19.2
4	116.8 ± 7.9	118.8 ± 16.6	123.3 ± 17.7
5	109.7 ± 19.6	108.1 ± 6.4	137.5 ± 20.0

Table 6-1. AChE activities of 5 brain areas of each of the Porton, RHA and RLA strains (5 rats of each strain). Activities are expressed as μMoles acetylthiocholine hydrolysed/min/gm protein (mean ± standard deviations). Strain means were compared for each brain area by Student's t-test. All comparisons showed non-significant differences, except area 5 (cortex) in which the RLA strain differed significantly from Porton and RHA strains in AChE activity ($P < 0.01$ and $P < 0.001$, respectively).

The strains showed no significant differences in AChE activity for brain areas except in the cortical sample. In this sample the RLA strain showed a significantly higher AChE activity than that of the other two strains. This discovery was especially interesting since no other differences in whole brain or brain areas AChE activity were noted between strains. The biological significance of this difference, however, may be in some doubt. The level of variation within groups is high and because it was possible to examine only relatively small groups of animals (5) for these determinations, the effects of this variation may not be fully controlled. Further to this reservation it is worthy of note that small dissecting errors that may occur when separating the cortex from 'mid-brain' area could have very significant effects on AChE measurements. Thus the caudate nucleus for example, an area of very

high AChE activity (Bennett et al, 1966), lies close to the floor of the cortex and a very small portion of this area contaminating the latter sample, may significantly increase the level of AChE activity measured there. One of the five cortical samples from the RLA strain rats showed a much higher activity than the other four and may be an example of such a contamination.

The lack of differences in AChE activities of whole brains and brain areas between the strains (with the possible exception of the cortical areas) may have great significance for the functioning of the cholinergic systems in the brains of these animals. The ACh concentrations of whole brains and certain brain areas of the Roman strains show significant differences (Chapter 5), thus the ratio of enzyme to substrate has also been changed by the selective breeding process.

CHAPTER 7

Discussion

7.1 The principle aim of this research was to investigate the role of central cholinergic mechanisms in the control of behaviour employing strains of rat selectively bred to possess different behavioural characters. It was hoped that by using strains with distinct and consistent differences, it might be possible to correlate some behavioural characters with central biochemical events more easily than if non-selected strains of animals were employed. It seems likely that when selective breeding for behaviour was performed, associated changes occurred in brain biochemistry. If some of these biochemical changes could be detected, either by direct chemical estimation or indirectly by the use of drugs, and shown to correlate with aspects of the behaviour it might be possible to make some useful observations or speculations about causal relationships.

First the significance of the behavioural differences measured in the undrugged animals of the three strains will be considered, followed by a discussion of the drug studies. Strain differences have been demonstrated in behavioural response to the anti-ChE drugs, the anti-ACh drugs and also to the non-cholinergic drug, d-Amphetamine. Although strain differences were not found in the activity of whole brain AChE, they were found in the ACh concentration of several different brain areas. Changes were also found in brain ACh concentration after the administration of the tertiary anti-ChE and anti-ACh drugs, which were strain dependent, but no changes were seen after the quaternary drugs or after d-Amphetamine. The significance of these behavioural differences in response to the drugs and similarly, the differences in

brain ACh will be discussed in turn, before the significance of correlations between brain and behaviour drawn from them is discussed. In particular, the support that the findings give to a theory of central cholinergic inhibition in the control of behaviour will be assessed.

Finally, other points of interest stemming from this work will be considered, including the potential usefulness of employing selectively bred strains, such as the Roman strains, in psychopharmacology and suggestions for further work in this field.

7.2 Normal Behaviour of the Strains

The behavioural experiments performed with the Roman strains revealed several features; first, it was confirmed that the Roman strains, selectively bred by Bignami (1965), demonstrate profound differences in the speed with which, and the extent to which, they acquire a conditioned avoidance response in a shuttlebox. The Porton strain rats demonstrated conditioning performance that fell between the extremes shown by the Roman rats. The RHA and RLA strains demonstrated strain differences of a type similar to those described by Bignami (1965) and Broadhurst and Bignami (1965), despite the use in this work of a shuttlebox and training techniques that were slightly different to the ones used for the original selection experiments. These differences included a shuttlebox of slightly different dimensions, a tone and shock presentation of shorter duration and a shorter, and constant, intertrial length. The avoidance learning of the RLA strain was, however, poorer than that described by Broadhurst and Bignami (1965), in fact, control levels of avoidance responding in this strain, even after several training sessions, were almost invariably zero. There was also a tendency for these rats to respond even more poorly as training continued, so that after several training sessions many rats failed to make any escape responses to shock. (Deterioration of performance of this type is also

seen with other strains of rat after repetitive shocks have been given in this way and is probably the result of stress). There are several reasons why poorer performance might have occurred in the RLA strain The first relates to the arrangements of shock presentations that were used; if the animal had not made an escape response within four seconds of shock presentation, the shock was automatically turned off. This was the maximum duration shock period possible with this equipment. In the selection experiments of Bignami (1965), the shock stimulus was maintained until an escape response was made, thus ensuring a response from all but the poorest subjects. The other possible reason for the unexpectedly poor responding of the RLA strain is one of pain reception. It is possible that this strain is less susceptible to shock stimuli, either by virtue of high skin resistance or because of a difference in pain perception (originating in the C.N.S.) and that the maximum shock stimulus used in these shuttlebox experiments may have been less reinforcing than that used by Bignami (1965). The latter reason is thought to be somewhat unlikely and is discussed further, in another context, in a later part of this discussion. The extra poor conditioning of the RLA rats was not considered to be a matter for concern, since the object here was simply to obtain strains which differed in behavioural responses and these animals contrasted considerably, with the RHA strain in their conditioning behaviour. The acquisition of avoidance by the RHA strain was also somewhat poorer than that reported for the strain by Broadhurst and Bignami (1965) but was nonetheless considerably better than that exhibited by the Porton strain. In this work the RHA females were significantly superior to the males which constrast with the findings of Broadhurst and Bignami (1965), who found the males to have shorter escape latencies (and therefore a tendency to condition more rapidly) than females, although the overall number of avoidance responses made

by each was about the same. These authors, however, examined conditioning during one session only, which corresponds to the first of the training sessions performed in this work and the sex differences seen here, only became evident in sessions 2 - 5. The Porton strain rats, which are not related to the Roman strain, demonstrated conditioned avoidance behaviour which fell between the extremes of the Roman strains and therefore made a useful behavioural control in this respect. Although, during early stages of training, many Porton strain rats demonstrated an occasional lack of escape responding, their overall performance resembled most closely that of the RHA rats, although they were somewhat poorer in speed of acquisition and in final trained performance. As in the RHA strain, there was a sex difference, the Porton females showing a tendency for superior conditioning over the males but in this strain the difference was not so pronounced. The three strains, then, provided a potentially useful range of conditioning performance for this work.

It has been suggested (Broadhurst and Wallgren 1964, for example) that performance in active shock avoidance test situations is related to the level of spontaneous locomotor activity exhibited by an animal, i.e., an animal which is active in a shuttlebox is likely to make avoidance earlier, and it was partly for this reason that a test of spontaneous activity was included in this work. Differences between the strains were shown which agreed with the suggestion that highly active animals tend to be conditioned rapidly in active avoidance tests, whilst less active animals are conditioned slowly. Thus the RHA rats show high exploratory activity, greater than Porton and RLA strain animals, and also maintain a higher level of spontaneous activity than the other two strains. The RLA strain shows a low level of all types of spontaneous activity but not significantly different from the activity of the Porton

strain rats. Thus high activity may predispose rats to good active avoidance acquisition but relatively low activity does not necessarily correlate with poor avoidance learning, since the Porton and RLA strains have similar activity levels but differ very significantly in their conditioned avoidance acquisition. Although comparisons of pure spontaneous activity of the Roman strains do not appear to have been reported before. Broadhurst and Bignami (1965), reported that the RHA strain rats show very much higher ambulation scores in an open-field test than their low avoidance strain counterparts. Although there is some controversy about the meaning of ambulation scores in the open-field (Whimbey and Denenberg , 1967) it may possibly be concluded that the high scores obtained by the RHA rats in the open-field represent high exploratory activity. It seems reasonable to suggest that the high level of exploratory activity seen in RHA rats contributes much to their rapid conditioning.

A behavioural difference between two of the strains that have been shown here is the difference in rates of extinction exhibited by the Porton and RHA rats. RHA rats took considerably longer to lose the conditioned response in the shuttlebox when the shock was withheld. Once again a high level of spontaneous activity in the RHA strain may have been an important factor, here contributing to the maintenance of the avoidance response. In a shuttlebox high levels of activity (avoidance responding) are rewarded and RHA rats, therefore have their, already high, activity level, further reinforced, so that during extinction training they take longer than Porton rats to habituate to the conditioned stimulus. It is likely that the rate at which an animal habituates to new stimuli is inversely proportional to its exploratory and general activity level. The possible role of cholinergic mechanisms in habituation, and therefore, perhaps, also in exploratory activity,

will be discussed later in the light of these and other findings.

7.3 The Drug Studies

The discovery of differences in response to drugs by the Porton and Roman strains may give the first clues to the underlying mechanisms responsible for the behavioural differences. If it can be shown that the strains respond in different ways to the administration of certain drugs and the mode of action of these drugs is at least partly understood, then it is reasonable to implicate the sites of action of these drugs in the control of the behaviour concerned. Clearly, such an implication is only suggestive of involvement in a neural network or chemical system, which may involve other components that are still unassessed, but none-the-less represents a step toward the understanding of the mechanism. The strain differences seen in this work, in behavioural responses to drugs affecting the ACh system may thus confirm the involvement in some, as yet, unspecified way, of this system in conditioned avoidance behaviour and also, perhaps, locomotor activity.

7.31 The Behavioural Effects of Anti-AChE Drugs

The first consideration will be the differences that were seen in the experiments that examined the effects of physostigmine on locomotor activity. The most obvious effect of physostigmine on all three strains, was behavioural depression which reduced, in particular, the exploratory phase of the activity recording period (Table 7-1). This may have been due to a selective action of the drug on exploratory drives or, as is probably more likely, represented the pharmacologically active phase of the drug, i.e., the AChE inhibition exceeded a critical level for depressing behaviour only during the first 30-40 minutes after injection after which sufficient reactivation of the enzyme had occurred, to allow a return to normal behaviour. Normal behaviour in this case was often

	Dose (mg./kg.)	Porton 1-45	Porton 46-90	RHA 1-45	RHA 46-90	RLA 1-45	RLA 46-90
Physostigmine	0.25	↑↓	↑	↓↓	→	↓↓	↓↓
	0.125	→	→	↓	→	↓	↓
Pyridostigmine	0.125	→	→	→	→	→	→

Table 7-1. Summary of physostigmine and pyridostigmine effects on spontaneous activity. 1-45 and 46-90, refer to portions of the recording period and therefore represent time periods (mins.). ↑↑ = increased activity observed in more than 2 of the 10-minute counts; ↑ = increased activity in 1 or 2 of the counts; → = no change; ↓ = decreased activity in 1 or 2 of the counts; ↓↓ = decreased activity in more than 2 of the counts. (Significant changes noted only).

preceded by a short period of enhanced activity, which, at first sight, is consistent with the work of Russell et al (1961), who reported facilitation of various types of behaviour with small reductions in brain AChE activity. Thus a point may have been reached during reactivation of the AChE when stimulation of activity replaced the depression. However, this explanation seems unlikely as; (i) no such stage was reached in the time immediately after injection, when presumably a similar level of AChE inhibition was passed, (although, the inhibition of AChE is probably much more rapid than the reactivation phase and stimulation, represented as a rise in activity, may be short-lived and hence not detected); (ii) no stimulation of activity was seen when low

doses of physostigmine were administered. It is thought more likely that these small increases in activity represent a delayed exploratory period. During inhibition of brain AChE rats are prevented from satisfying exploratory drives and as the effects of the drug subside they complete their exploration of the box. A similar effect has been seen after non-sedative doses of a barbiturate in rats. Here also, the depression phase was followed by hyperactivity, (this author, unpublished observations).

The RHA strain showed the most profound depression after physostigmine. The depression was greatest during the exploratory phase after which there was a tendency for the hyperactivity already described. By contrast, the RLA strain animals showed depression of a less extreme type but the depression lasted for a much longer period of time and these rats never exhibited a hyperactivity phase. The Porton strain showed the least response to physostigmine, with short-lived depression, followed rapidly by hyperactivity, but this only at the highest dose used. Physostigmine inhibits AChE in all parts of the body and changes in behaviour seen as a result of its administration should not be assumed to be necessarily central in origin, however, experiments performed here in which the quaternary anti-ChE drug, pyridostigmine, was administered, suggest that the effects seen after physostigmine probably were the result of drug effects on the C.N.S. Pyridostigmine, which is not thought to pass the blood-brain barrier, produced no effects on spontaneous activity in any of the strains, at a dose which might be expected to produce a level of peripheral AChE inhibition, equivalent to that produced by a dose of physostigmine of approximately, 0.19 mg./kg. (This dose is about half-way between the two doses of physostigmine actually used, viz., 0.25 mg./kg. and 0.125 mg./kg.). These doses of physostigmine and pyridostigmine are based on the comparative potencies for the drugs implied by the work of Leadbeater and Gordon (personal communication

1973), after the observation that the maximum doses of these drugs that could be given to rats without producing toxic signs, were 0.1 mg/kg. and 0.075 mg/kg., respectively.

As with the spontaneous activity experiments in which physostigmine was given, a depression of behaviour was also seen when this drug was given to rats training in a shuttlebox. A summary of the effects of physostigmine on shuttlebox learning in the three strains is shown below in Table 7-2.

Dose (mg/kg.)	Porton		RHA		RLA	
	Avoids.	Fails.	Avoids.	Fails.	Avoids.	Fails.
0.125	↓↓	↑↑	↓↓	↑↑	→	↑
0.06	↓	↑	↓↓	→	→	↑
0.03	→	→	→	→	→	↑

Table 7-2. Summary of physostigmine effects on shuttlebox training. Avoids. = Conditioned avoidances; Fails. = failure to escape; ↓↓ = decreased responses in more than 2 out of the 5 training sessions; ↓ = decreased responses in 1 or 2 sessions; ↑↑ = increased responses in more than 2 training sessions; ↑ = increased responses in 1 or 2 training sessions; → = no change, (significant changes noted only).

The effect of physostigmine at the highest dose used was to depress conditioned and unconditioned behaviour to a high degree in all three strains. The severity of these effects may be judged from the fact

that not only was avoidance learning impaired but also that most unconditioned responding (escape behaviour) was also inhibited in the Porton and RHA rats, so that they resembled the RLA rats in their shuttlebox performance after this dose. The avoidance learning of the RLA strain rats was not altered from zero, but their escape behaviour was made even worse by physostigmine. The degree of change in the RLA rats was not as great as in the other strains but clearly, these animals normally show poor responding which already approaches the minimum. It is important to note that although in all strains the block of responding at this dose is severe, it does not appear to be simply a 'non-specific, toxic effect', affecting all types of behaviour. When this dose of physostigmine was administered to rats in the spontaneous activity experiments, only minor depression of activity was seen in the Roman strains and no change at all in the activity of the Porton strain. Thus these animals were not suffering a general behaviour depression at this dose, but may have been suffering a specific block of unconditioned and conditioned behaviour. (It was observed earlier that low sponataneous activity need not necessarily mean poor avoidance behaviour. The present observations allow the additional observation that poor avoidance behaviour need not be associated with low spontaneous activity). The possible explanation for the block of conditioned avoidance in terms of a cholinergically controlled, 'response inhibition', will be discussed later in this chapter. The effect of physostigmine on the RHA and Porton strains was dose dependent. At the second dose tested, the Porton strain rats showed simultaneous impairment of conditioned and unconditioned behaviour but to a lesser extent, than was produced by the high dose, whereas the RHA rats clearly exhibited a severe block of avoidance learning whilst their escape behaviour was normal. This last finding supports the previous contention that the effects of physostigmine seen in these experiments constitute a specific block of shuttlebox responding rather than a non-

specific behavioural depression. The reason for the strain difference exhibited between Porton and RHA rats is not clear, and is especially difficult to understand since the avoidance responding of the Porton strain was reduced to a lesser extent than that of the RHA strain at the same dose, but show a significant reduction in escape behaviour. Neither of these strains was significantly affected by the lowest dose of physostigmine (0.03 mg/kg.) but the RLA rats were equally affected by all the doses employed. It is difficult to make comparisons between the effects seen with RLA rats and those with the other two strains, since negligible avoidance learning is normally seen in the RLA strain. All that can be stated at this stage is that the unconditioned responding of RLA rats is more sensitive to the effects of AChE inhibition than that of the other two strains studied, and that as the strain already exhibits an almost minimal performance in this test, the appearance of a dose dependent effect is unlikely. That the behavioural changes seen, arise from changes produced in the C.N.S., was confirmed by the experiments in which pyridostigmine was substituted for physostigmine. No significant behavioural changes were seen in the shuttlebox performance of any of the strains after pyridostigmine. (Pyridostigmine was given in a dose (0.125 mg/kg.) chosen to produce a peripheral inhibition of AChE, equivalent to that of a dose of physostigmine in excess of the highest dose (0.125 mg/kg.) used in these experiments).

The doses of physostigmine which have been used in these experiments may be expected to produce a fairly extensive inhibition of brain AChE, but because this drug is a reversible inhibitor of ChE, reactivation of the enzyme occurs rapidly. For this reason high levels of AChE inhibition are produced for relatively short periods of time and no periods of steady levels of inhibition are seen. This means that the time between injection of drug and behavioural test must be kept constant

to make experiments comparable. Accurate chemical estimation of ChE inhibition in animal tissue after treatment with reversible anti-ChE agents is very difficult since reactivation of the enzyme occurs in the tissues during tissue preparation and estimation and no completely satisfactory methods are available at the moment. Attempts were not made in this work to estimate the levels of AChE inhibition that were present during the behavioural experiments with physostigmine and so direct comparison of this data with some of the other behavioural work in which irreversible anti-ChE agents were used and AChE estimations made of the brains, is not possible, (however, some of the effects of reduced AChE activity in the brain can, of course, be seen from the ACh estimations made, after treatment with physostigmine). If the behavioural effects are compared with those of Russell et al, (1961), for example, it may be assumed that brain AChE inhibition was in excess of 60-70% in some of the shuttlebox experiments performed here. These authors, and others, have demonstrated that significant deterioration in behavioural performance of rats, in various avoidance tests, is not seen until the critical level of 60-70% inhibition is reached. Thus in the shuttlebox experiments brain AChE in the RHA and Porton rats after doses of 0.125 mg/kg. and 0.06 mg/kg. were given, was probably less than 30% of normal and after 0.03 mg/kg., greater than this level. It is interesting to note that in none of the experiments involving physostigmine was an enhancement of a behavioural activity seen (with the exception of some hyperactivity seen in a few of the spontaneous activity experiments, in which the likelihood of the effect being due, directly, to physostigmine, has been discounted). Enhanced performance of various behavioural parameters has been described (Cox and Tye, 1973, and Russell et al, 1961, for example) after small doses of anti-ChE agents but has not been described for shuttlebox avoidance learning. Perhaps, therefore, the type of response measured, is an important factor in this effect.

The tolerance exhibited by animals to chronically reduced AChE activity (Russell et al, 1972; Chippendale et al, 1972; Barnes and Denz, 1954) may have a parallel in some of the shuttlebox experiments performed here with RHA rats. Although in these experiments physostigmine was administered for a relatively short period of time (4 days), some of the RHA rats showed a small improvement in avoidance responding and a larger decrease in escape inhibition on the third and fourth days of training with the drug. This was most evident after the high dose (0.125 mg/kg.; see Figure 3-8) and possibly occurred also, but to a lesser extent, after the lower dose (0.06 mg/kg.; see Figure 3-11). Whether this effect was due to the development of a pharmacological tolerance to the drug itself or to the development of behavioural tolerance to low AChE cannot be decided without assessment of the brain AChE activity present during each training session. The development of either type of tolerance would be surprising under these circumstances. The drug effects were relatively short lived and treatment was only repeated four times altogether; this is compared to the work of the authors quoted, in which the irreversible anti-ChE agents were administered in such a way as to produce a constant level of enzyme inhibition for several days or even weeks at a time.

In all the shuttlebox experiments with anti-ChE agents a return to, or a strong tendency towards a return to, control levels of responding when the drug was withheld, (on the last session) was always seen.

7.32 *The Behavioural Effects of Anti-ACh Drugs*

Having discussed some of the implications of AChE inhibition, i.e., enhanced ACh activity, in behavioural experiments the effect of reducing the action of ACh by administering anti-ACh drugs will be considered in this context. As has already been discussed, the work of other authors indicate that the administration of anti-ACh drugs to rats

in behavioural situations, produces enhanced or impaired performance according to the type of test used and the time of administration of the drug. It might have been anticipated that in the present work, in which the drug was given, before the test procedures began that enhancement of performance and hyperactivity would be seen after the administration of a centrally acting, anti-ACh drug. Such effects were, indeed seen, with all strains exhibiting hyperactivity in the spontaneous activity experiments and facilitated avoidance conditioning in shuttlebox experiments after the administration of the anti-ACh drug, NEPB.

Although rats of the three strains all demonstrated hyperactivity (cf. Abood, 1959), the RLA strain showed the greatest increases in locomotor activity (Table 7-3).

	Dose (mg/kg.)	Porton		RHA		RLA	
		1-45	46-90	1-45	46-90	1-45	46-90
NEPB	1.0	↑	→	↑	→	↑↑	↑↑
	2.0	↑	→	↑	↑	↑↑	↑↑
NEPB MeI	1.0	→	→	→	↓	→	→

Table 7-3. Summary of NEPB and NEPB MeI effects on spontaneous activity. 1-45 and 46-90, refer to portions of the recording period and represent time (mins.). For key to symbols see Table 7-1.

Both exploratory and normal activity levels were increased but the effect did not appear to be dose dependent, approximately the same degree of hyperactivity was produced with each dose used. Exploratory and normal activity levels of the RHA rats were also increased at the high dose (2.0 mg/kg.), but to a lesser extent, and the low dose (1.0 mg/kg.)

produced only a short-lived increase in locomotor activity. The Porton strain animals showed the least change after NEPB, which was a short-lived increase in exploratory activity at each dose used. Thus, strain differences were once again exhibited in response to a drug affecting central cholinergic systems. The order of strain sensitivity to NEPB contrasted with that for the anti-ChE agents; thus RHA rats, which showed greatest sensitivity to increased ACh levels, after physostigmine, showed less sensitivity than the RLA strain to an anti-ACh drug, which effectively reduced central ACh activity. The behaviour of the undrugged Porton strain in the tests employed here, it will be remembered, lies between the extremes of the Roman strains but its response to anti-ACh and anti-ChE drugs lies outside the extremes of response shown by the other two strains.

It has been seen that although reduced activity (compared to normal), induced or natural, is not necessarily correlated with poor conditioned avoidance; hyperactivity usually facilitates learning. Thus it was seen in the experiments performed with NEPB and shuttlebox learning, that the overall effect was one of enhanced responding in all strains (Table 7-4).

	Dose	Porton		RHA		RLA	
	(mg/kg.)	Avoids.	Fails.	Avoids.	Fails.	Avoids.	Fails.
NEPB	1.0	↑	→	↑	→	↑	↓
NEPB MeI	1.0	→	→	↓	→	→	↑

Table 7-4. Summary of NEPB and NEPB MeI effects on conditioned avoidance. Key to symbols as for Table 7-2.

The Porton strain showed a marked enhancement of avoidance responding in later drug sessions whilst the RHA rats showed improvements which although significant, were of a smaller degree, because this strain's performance was normally very high and this, therefore, tended to limit the possibility of further improvement in responding. Perhaps, the most striking result from these experiments with NEPB, was the avoidance learning demonstrated by the RLA strain. Not only was the escape behaviour improved but avoidance behaviour was also seen, for the first time with the RLA strain. Although significantly improved over control performance, avoidance responding only reached approximately 10% of the highest level in session 3 and 4, and fell back, to almost zero, when the drug was withheld in session 5. It has already been shown that the dose of the anti-ACh drug used, produced marked hyperactivity in the RLA strain and so it is possible that a simple measure of locomotor activity was obtained in the shuttlebox. If these rats were very hyperactive it is possible that a large number of shuttlebox crossings were made, spontaneously throughout the session and those which occurred during the tone presentation, were recorded as avoidance responses. Unfortunately, the shuttlebox which was used in this work did not provide a count of intertrial responding, which might have permitted a better analysis of this problem to be made. The level of responding may be low enough to be explained in terms of fortuitous, random crossings and the observation that these responses return to near zero when the drug is not given, appears to further support this possibility. Rech (1968) also demonstrated facilitation of shuttlebox performance after administration of anti-ACh drugs which was not maintained into the undrugged state, but Barrett, Laith and Ray (1972) showed that the lack of transfer between states may be caused by the sudden withdrawal of the drug. These authors produced facilitation with hyoscine, which was maintained into the undrugged state by gradual withdrawal of the drug, i.e., the dose of drug was gradually

reduced over several sessions.

In some previous work using the drug, NEPB and shuttlebox behaviour of the Porton rats (Brimblecombe and Buxton, 1972) it was possible to measure intertrial activity alongside avoidance responding and draw conclusions about their possible relationship. Although the dose of drug involved in these experiments was very much larger than that used in the present work and also the design and use of the shuttlebox slightly different, similar factors were clearly involved. By calculating the probability of responses occurring during tone presentation or intertrial periods, it was possible to show that the responses made were not distributed according to a random pattern, as would be expected if the avoidance responses were little more than fortuitous wanderings, but tended to be accumulated in the relatively shorter, tone presentation period. Thus in these experiments, at least, true avoidance learning was taking place although the animals were also hyperactive. It is not possible, however, to be so certain that enhanced avoidance learning per se, was produced in all the shuttlebox work here described. The Porton and RHA strains show a high retention of conditioned avoidance behaviour in session 5, after drug withdrawal, showing that true learning took place under drug effects, and, in any case, avoidance responding at the levels seen in these strains could only result some hyperactivity alone, if very high levels of activity were present, higher, in fact, than those recorded in the spontaneous activity experiments. If very high levels of avoidance are attained with the drug, the effects of sudden drug withdrawal are clearly less deleterious than when respondis low. It may be assumed therefore, that enhancement of avoidance learning was seen in the Porton and RHA strains after administration of NEPB and possibly, an enhancement was seen in the RLA strain.

In order to check that the effects produced by NEPB on behaviour

were central effects, experiments were performed with all the strains, using the quaternary analogue of NEPB, NEPB MeI. In the first experiments with this drug an apparent depression of locomotor activity was recorded with Porton strain rats which was later shown to be a recording artifact. Quaternary anti-ACh compounds, as a rule, possess greater anti-ACh activity, peripherally, than their tertiary counterparts. Thus, it appears that the dose of NEPB MeI used in these experiments (1.0 mg/kg.; also equal to the dose of NEPB used) produced peripheral effects, which included severe drying of the foot-pad skin of the rats. This probably had the effect of increasing the electrical skin resistance of the rats' feet to a level which prevented reliable recording of activity boxes dependent upon good electrical contact between the animals' feet and the cage bars. This problem was overcome very simply, by smearing the bars of the activity cage with a saline gel, for all further work in which anti-ACh drugs were used. After taking this precaution the differences previously seen in locomotor activity after NEPB MeI disappeared, but the finding, of course, questioned the validity of the earlier experiments in which NEPB had been given to rats in these activity cages. Although increases in activity were recorded after NEPB, the hyperactivity may have served to mask some recording losses equivalent to those seen after NEPB MeI.

However, a repeat experiment, therefore, using Porton rats, NEPB (1.0 mg/kg.) and saline gel on the cage bars produced results that did not differ from those of the original work.

When NEPB MeI was administered to rats in shuttlebox experiments a reduction in behavioural performance was also seen. All the strains showed a tendency toward poorer conditioning than controls, and in both Roman strains, significant reductions in avoidance responding were seen. Since this was opposite to the effect to that seen with NEPB, it seemed

possible that, increased skin resistance may have played a part in raising pain thresholds to shock in the shuttlebox. The experiment performed to compare pain thresholds to shock after administration of the two anti-ACh drugs, confirmed this. It is therefore concluded that the poor responding seen after NEPB MeI was probably due to a reduction in the painfulness of the unconditioned stimulus, which was thus less reinforcing than usual. It was also concluded that at the dose chosen, NEPB did not produce a measureable change in pair receptivity to shock and so there was no reason to doubt the results obtained in those experiments. Thus, despite the complicating factors present in the NEPB MeI experiments, it is reasonable to assume that the changes produced to spontaneous activity and shuttlebox avoidance by NEPB, were the result of central drug effects and presumeably, therefore, the result of central anti-muscerinic activity. Enhancement of learning after the administration of anti-muscarinic drugs supports the findings of Bignami et al (1965), Rech (1968), Barrett et al (1972) and Brimblecombe and Buxton (1972). The possible significance of the strain differences to NEPB will be further discussed in the light of other drug/strain differences, and the finding that NEPB extends extinction times in a strain dependent manner, later in this chapter.

7.33 Manipulation of Central Adrenergic Systems

The drug effects discussed so far have all involved direct manipulation of the cholinergic system. The appearance of strain differences in response to these drugs has been noted and it has been implied that the differences exhibited by the strain in behaviour and drug response may suggest a causal relationship between the cholinergic system and behaviour. The aim of this thesis was principally, to look for correlations between behaviour and the cholinergic systems of the brain but, of course, other systems may be expected to interact with the cholinergic

system in the control of behaviour. In order to study, therefore, the possible interaction of cholinergic and adrenergic systems, a series of experiments was also conducted in which the C.N.S. stimulant, d-Amphetamine,was given alone, or in combination with NEPB. 0.1 mg/kg. of d-Amphetamine given alone, produced increased locomotor activity in all the strains and strain differences were observed in the extent of this response.

Dose	Porton		RHA		RLA	
(mg/kg.)	1-45	46-90	1-45	46-90	1-45	46-90
d-Amphetamine 0.1	↑	↑	↑	↑↑	↑↑	↑↑

Table 7-5. Summary of d-Amphetamine effects on spontaneous activity. For key to symbols see Table 7-1.

The fact that amphetamine produced significant effects implies that adrenergic mechanisms are involved in the manifestation of locomotor activity and further, that the differences may indicate that there are strain variations in adrenergic as well as cholinergic mechanisms.

The Roman strains stand apart from the Porton rats in their response to d-Amphetamine, just as they did in their responses to NEPB and physostigmine, showing a greater response to these drugs than the Porton rats which show only low responses. This may suggest a difference between these strains which is a function of their different origins and not necessarily of their selective breeding or behaviour. Differences were also shown to exist between the Roman strains in their response to d-Amphetamine, with the RLA rats showing the greatest hyperactivity, and particularly increased responding during the exploratory phase of the experiment. This appears to contrast with the findings of Robbins and Iverson (1973)

in which d-Amphetamine caused a decrease in exploratory behaviour but facilitated locomotor activity. These authors employed a test situation in which it was apparently possible to measure, simultaneously, exploratory behaviour and locomotor activity as separate entities, but unfortunately, it appears that the design of the test was such that almost inevitably an increase in one parameter would bring about a decrease in the other.

The facilitating effects of d-Amphetamine on various types of active conditioned avoidance have been demonstrated by several authors (Hearst and Whalen, 1963; Rech, 1966; Stein and Wise, 1970) and have also been shown in this work (Table 7-6).

Dose (mg/kg.)	Porton		RHA		RLA	
	Avoids.	Fails.	Avoids.	Fails.	Avoids.	Fails.
d-Amphetamine 0.1	↑	→	↑↑	→	↑	↓↓

Table 7-6. Summary of d-Amphetamine effects on conditioned avoidance. Key to symbols as for Table 7-2.

It is often assumed that the facilitation is due in large part to the hyperactivity produced, which provided the facility for greater exploration and, in particular, earlier opportunity to learn in avoidance tests in the way that was described for the effects of NEPB. The RLA rats again showed improved conditioned avoidance but the improvement was mainly confined to a reduction in the number of unconditioned response failures, so that with d-Amphetamine most rats were able to escape the majority of shocks presented. The low level of conditioned avoidance responding, was dependent on the presence of drug as shown by the return to zero

avoidance responding when the drug was withheld in the final session. Gradual drug withdrawal may have permitted some transfer to the undrugged state (Barrett et al, 1972), although it is surprising that greater facilitation with d-Amphetamine was not seen with the RLA strain, Bignami (1965) and more recently, Coyle, Wender and Lipsky (1973), described a facilitation of shuttlebox learning in RLA rats after d-Amphetamine greater than was seen here but with a larger dose of d-Amphetamine. However, these authors conclude that the avoidance scores of the RLA rats were probably not conditioned responses but rather a measure of their hyperactivity. Davies (personal communication, 1973), trained RLA strain rats in a pole-jump avoidance test, producing facilitation of the response with a dose of d-Amphetamine close to the dose used in these experiments. Bignami (1965) in these experiments, employed a shuttlebox technique similar to the one used during the selection experiments, in which low level avoidance responding occurred with RLA rats without drug, so it is possible that the differences in technique are responsible for the differences in drug response seen here. Similarly, Davies (1973) showed that RLA rats were capable of learning pole-jump avoidance without drug, although at a slower rate than other rats. Thus both these reports appear to show enhancement of learning, whilst the RLA rats in the experiments reported here showed, initially, negligible avoidance and so initiation rather than enhancement of avoidance responding was the requirement. Avoidance responding in the Porton and RHA strain rats after d-Amphetamine, however, was significantly enhanced and in the case of the RHA rats to a very high level of responding. As with the NEPB experiments, it can be concluded that enhancement of responding, which was maintained after drug withdrawal, was seen in RHA and Porton rats after administration of d-Amphetamine. A similar type of effect was seen, although to a much lesser degree, with the RLA strain.

The results of the experiments in which combinations of NEPB and d-Amphetamine were given to rats will be discussed briefly here and more fully later in the context of theories of interacting chemical systems in the C.N.S.

Doses of NEPB and d-Amphetamine were chosen which, given separately, produced no change in either spontaneous activity or conditioned avoidance behaviour and these were given simultaneously. It was hoped that if cholinergic and adrenergic systems have reciprocal actions in controlling behaviour, the effect of giving both drugs together may demonstrate such a relationship by producing a behavioural change. The effect of the combination of drugs on spontaneous activity was to increase significantly, part of the locomotor activity period but not affect the exploratory period. These experiments were performed using Porton strain rats and when given effective doses of d-Amphetamine and NEPB separately, these animals show most change in the earlier parts of the activity period, during the exploratory phase. The significant changes produced after the drug combination strongly imply an interaction between cholinergic and adrenergic systems in the control of locomotor activity but also there was a delay in the time to effect which was not seen when larger doses of these drugs were given separately. These findings apparently confirm those of Carlton (1963) although using a different type of behaviour and larger doses of drugs. The latter difference may explain the relatively small effect seen in the present work as being due to small doses of d-Amphetamine (0.1 mg/kg. compared to Carlton 0.25 mg/kg.) and NEPB. The same doses of the two drugs were also given in combination to rats of the three strains training in a shuttlebox. Facilitation of avoidance conditioning was seen in all strains. The facilitation was highly significant in the Porton and RLA rats, both demonstrated responding which was approximately similarly to that shown after single doses of NEPB or d-Amphetamine in the earlier experiments. The effect on RHA conditioned

avoidance was not significant and the percentage enhancement was less because, as in previous studies, control responding is normally high. These results strongly suggest interaction between adrenergic and cholinergic systems in the control of conditioned avoidance behaviour and spontaneous activity.

7.4 The Biochemical Estimations

7.41 Normal Cholinergic Activity of the Strains

It has been established that strain differences exist in behaviour and in the behavioural response to several types of drugs, the attention will not be turned to differences discovered in brain biochemistry and chemical response to the same drugs. Estimation of the ACh concentration in the whole brains of animals from the Porton, RHA and RLA strains revealed significant differences. It was found that brain of RHA rats possessed significantly less ACh than those of the RLA rats (a difference of approximately, 16%) and the ACh concentration in the brains of Porton rats, although not significantly different from that of either Roman strain, fell between the extremes of the other two. Estimation of AChE activity, however, revealed no significant strain differences, in whole brains or brain areas, with the exception of the cortical area. Here a significant difference was found between the Roman strains, the RLA rats having slightly higher activity in this region. (It has been suggested in Chapter 6, and support for the suggestion will be given when drug effects on brain ACh are discussed, that this difference may not be biologically significant). The strains therefore, not only possess varying concentrations of ACh, but also variations in the ratio of enzyme to substrate in the whole brain and also probably in the brain areas. Thus the simple correlation can now be made, that high ACh concentration relative to AChE activity is associated with poor avoidance behaviour

and reduced spontaneous activity levels and conversely, low ACh concentration relative to AChE activity is associated with fast avoidance conditioning and high spontaneous activity levels. The Porton strain animals fall between the Roman strains in both their behavioural performance and in their ACh to AChE ratio. Although the difference in ACh concentrations between Roman and Porton strains are not significantly different, the Porton animals have a brain ACh concentration most like that of the RHA strain which they also most resemble in their conditioned avoidance behaviour. The ACh and AChE estimations made of brain areas permit a closer analysis of these differences to be made. The RLA rats possess greater concentrations of ACh than the RHA, in all the brain areas examined except the mid-brain region and also differ from the Porton rats in their hypothalamic and cortical concentrations of ACh. The relative quantities of ACh in brain areas within each strain compare well with the findings of other workers (Macintosh, 1941; Quastel, 1962), thus the greatest quantity is seen in the hypothalamus, whilst the large, mid-brain region, which included the caudate nucleus and most of the brain stem, both areas known to contain high concentrations of ACh, contained the next highest concentration, (Quastel, 1961). The medulla and cortex contained approximately equal concentration, whilst the cerebellum contained only very small quantities which were sometimes barely measureable.

The relative activities of AChE measured in the brain areas, agree with those measured by other workers (Bennett, Diamond, Morimoto and Herbert, 1966) and show approximately the same distribution as ACh. It is interesting that although the selective breeding experiments appear to have changed ACh concentration in a fashion that correlates with the changes in behaviour, AChE activity remains relatively unaffected. This implies that ACh and its metabolic enzyme are not genetically linked as might have been anticipated. AChE is present in many areas of the body,

including areas where there is apparently no ACh and so may have secondary roles as yet not understood. For this reason, the genes, responsible for AChE may not be restricted to the same chromosomal loci as those for ACh and would tend, therefore, to be separated when selection pressure was imposed on one of the components. The selective breeding experiments of Roderick (1960) and Bennett et al (1960, 1964) are relevant in this context. These authors selectively bred animals for brain ACh concentration or AChE activity. It was found that when ACh was the selection character, the resulting strains possessed high or low ACh, according to the direction of the selection, but retained the ratio of ACh to AChE of the original stock. Thus there was apparently a demonstration of genetic linkage. However, in experiments where the enzyme activity was the criterion for selection, ACh levels remained constant, thus altering the ratio of enzyme to substrate. Since AChE is not only widely distributed in the body but is also present in quantities that may be in excess of minimum requirements (implied by the work of Russell et al, 1961; Barnes and Denz 1954, and others) changes in the ratio of AChE to ACh, such as those described here, need not be relevant in terms of significant alteration to cholinergic activity at synapses. If the difference in ACh concentration between the RHA and RLA strains is large enough to make a significant difference to the functioning of cholinergic synapses in the C.N.S., then variations in the response of the Roman strain rats, and possibly also the Porton rats, to cholinergic drugs, would be expected. Such variation has been seen in behavioural changes and in changes of ACh concentration.

7.42 ACh Concentration and Drugs

A summary of the effects of anti-ChE and anti-ACh drugs on brain ACh in the three strains is shown in Table 7-7. The changes were found to be dose dependent and tended to be restricted to certain areas. The

	Dose	Porton					RHA					RLA				
	(mg/kg.)	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Physostigmine	0.5	↑	→	→	↑↑	↑↑	NOT TESTED					NOT TESTED				
	0.25	↑↑	→	→	↑↑	↑↑	↑	↑↑	→	→	→	↑↑	↑↑	→	↑	↑
	0.125	↑	→	→	→	→	↑↑	↑	↑	→	→	→	→	→	→	↑
NEPB	1.0	→	→	↓↓	→	↓	→	→	→	↓	↓↓	→	→	→	↓	↓↓

Table 7-7. Summary of effects of physostigmine and NEPB on brain ACh concentration in 5 brain areas. 1 = medulla; 2 = cerebellum; 3 = hypothalamus; 4 = 'mid-brain'; 5 = cortex. Arrows denote significant changes from control: ↑ = increase ($P < 0.05$); ↑↑ = increase ($P < 0.01$ or $P < 0.001$); → = no change; ↓ = decrease ($P < 0.05$); ↓↓ = decrease ($P < 0.01$ or $P < 0.001$).

most commonly changed areas were the medulla, 'mid-brain' and cortex and the area least affected by both drugs, was the hypothalamus. As expected the effects seen after physostigmine were large increases in ACh due to the inhibition of AChE, permitting the accumulation of released ACh. At the high dose (0.25 mg/kg.) increases in ACh were very high in some areas (e.g. over 100% in the medulla of all strains) and overall effects were greatest at this dose in the RLA strain which already possesses the highest ACh levels of all the strains. At the lower dose (0.125 mg/kg.), however, the RHA rats were still demonstrating a much increased ACh concentration which correlated well with the depression of behaviour seen at this dose. The RLA strain, however, at this dose showed changes in ACh in one brain area only and the Porton strain, no changes, although all of the strains exhibited behavioural changes at this and lower doses.

It is important to note at this point, that there was a difference between the Roman strains, in the response of the cortical regions to physostigmine (0.125 mg/kg.). This fact has an important bearing on the earlier discovery that cortical AChE activity between the Roman strains may be different. RLA rats were seen to have a slightly higher AChE activity but the importance of this finding was in doubt. If the higher AChE activity of this area of the brain in RLA rats was sufficient to reduce the significance of high ACh concentration in this strain, it is possible to make a prediction. If the AChE activity of the RLA strain was increased with the ACh concentration by a biologically significant amount, then the ratio of enzyme to substrate will be small (perhaps close to that in the RHA rats). Thus the effect of a small dose of an anti-ChE agent, in increasing ACh levels, would be less than if the AChE activity had been relatively unaffected, i.e., the enzyme might be expected to retain 'control' of the substrate even when a small percentage of the enzyme is inhibited. When 0.125 mg/kg., physostigmine was given to Roman rats, the cortical area of the RHA strain showed no change in ACh concentration but the RLA showed a significant increase. This appears to indicate that the ratio of AChE to ACh in the RLA strain is large (larger than in the RHA) despite the increased AChE levels measured for this strain.

Further evidence for a high ratio of ACh to AChE in the RLA strain comes from the spontaneous activity experiment with physostigmine (0.25 mg/kg.). The recovery of normal spontaneous activity in this strain was very delayed compared to that of the RHA rats in the same experiment. Presumably, although reactivation of AChE occurred at the same rate after physostigmine treatment, in both strains, sufficient AChE was released for normal behaviour in the RHA strain because of the relative excess of enzyme available under normal conditions in this strain. It may therefore, be justified to refer to 'high' and 'low' ACh levels with reference

to the AChE activities, of the cortices of the RLA and RHA strains, in the same manner as for whole brains.

The technique of extracting and estimating ACh concentrations that has been used, is not sufficiently subtle to detect very small changes that may well occur at these doses. It is however worth pointing out that the doses of physostigmine used here were realistic in the sense that they were similar to the ones used in the behavioural experiments. Crossland and Slater (1968), when examining the effects of physostigmine on whole brain ACh concentration in rats, employed a dose of 0.75 mg/kg., which is close to the LD_{50} for this drug and certainly unrealistic in behavioural terms. It has been observed that not all brain regions were affected to the same degree after a given dose of physostigmine. In particular, the hypothalamus, was barely affected at all, only showing an increase in ACh in the RHA rats after the low dose of physostigmine. This was true even when adjacent areas demonstrated large increases in ACh. The hypothalamus is an area of high concentration of AChE and ACh and it is possible that the high endogenous AChE maintained control over its substrate at the doses employed. The areas most affected by physostigmine include the areas most likely to be involved in the control of locomotor activity and learning behaviour, i.e., the cortex and mid-brain structures. Much attention has been given by various workers, to the hippocampus as a likely centre for certain types of learning behaviour. Lesions in this area produce hyperactivity and enhance shuttlebox learning in rats (Teitelbaum and Milner, 1963; Isaacson, Douglas and Moore, 1961). The hippocampus is rich in ACh and AChE and is contained in the 'mid-brain' portion of these studies, so may be a candidate for a cholinergic inhibitory centre controlling learning behaviour. Thus, ablation of the hippocampus (Isaacson et al, 1961) facilitates certain types of learning, and stimulation by treatment with physostigmine causes inhibition of the same type

of learning. Weiner and Deutsch (1968), however, have injected physostigmine directly into the hippocampus and shown facilitation of a visual discrimination test in rats.

Confirmation that pyridostigmine has little or no effect on brain AChE was obtained in the experiments in which rats treated with a large dose of the drug failed to produce significant changes in brain ACh.

The administration of NEPB produced large reductions in detectable ACh (Table 7-7) in all the strains and confirmed similar findings for other anti-ACh drugs (Crossland and Slater, 1968) but no changes were seen after NEPB MeI, confirming that this drug's actions were restricted to the P.N.S. ACh concentrations were significantly reduced in the cortical areas of all strains after NEPB and to the greatest extent in the Roman strains. The Porton strain experienced a significant decrease in hypothalamic ACh, the only occasion, in all these strains, when this brain area showed a change in ACh concentration in response to the drug. It is difficult to see why this should have occurred here. Generally, the ACh reductions correlate with the behavioural changes seen with the drug, in as much as both Roman strains showed the greatest behavioural responses to NEPB and also the greatest ACh changes. There is little correlation between the change in ACh level and behavioural change in the Roman strains, since ACh changes were similar, but behavioural changes quite different. This may be due to the differences in endogenous ACh between the strains. Thus although similar percentage changes in ACh were seen, the changes in the ratio of ACh and its antagonist, if such a system exists, may not have been similar.

It is worthy of note, that significant changes in ACh level were detectable after a relatively small dose (1.0 mg/kg.) of NEPB, the same dose, in fact, as the one used in all the behavioural studies and a more meaningful dose, it is suggested, than doses of atropine (25 mg/kg.)

used in other work (Crossland and Slater, 1968). (The relative potency of NEPB to any atropine, in producing central effects is 1 to 3-6 respectively, (Brimblecombe and Green 1968), therefore a comparable dose of atropine to produce similar changes would be 3.0-6.0 mg/kg.)

Administration of d-Amphetamine did not produce any significant changes in brain ACh levels although behavioural changes were seen after its administration to rats of all three strains. The biochemical origin of these changes, therefore, must be outside the cholinergic system of the brain. However, the presence of strain differences in the behavioural effects may either imply differences in catecholamine levels or, indirectly, reflect the ACh differences already observed, assuming there is a cholinergic-adrenergic relationship. A combination of both effects may be more likely.

The participation of catecholamines in the control of certain types of learning behaviour has been demonstrated. Depletion of catecholamines has been shown to decrease conditioned avoidance responding (Moore, 1966), whilst monoamine oxidase inhibitors restore amine levels and conditioned avoidance (Moore and Rech, 1967). Inhibition of catecholamine synthesis, with α-methyl-p-tyrosine, impairs avoidance conditioning in parallel with the reduction in amines, whilst d-Amphetamine reverses the response decrement (Hanson, 1967). Coyle et al (1973) examined the Roman strains for differences in catecholamine levels by comparing the activities of the enzymes responsible for amine synthesis. The activities of tyrosine hydroxylase, depamine-β-hydroxylase and phenylethanolamine N-methyl transferase were found to be slightly lower in the RLA than in the RHA strains but turnover of noradrenaline was identical. The authors concluded that although there were some significant differences in amine activity between these strains, they were not sufficient to explain the behavioural differences.

7.5 Cholinergic-Adrenergic Interaction and Cholinergic Inhibition

7.51 A recurring theme in the work described here is the relationship between cholinergic activity, measured in terms of ACh concentration, and the level of behavioural performance. This was seen first when endogenous ACh concentrations in the three strains were compared. The RHA, Porton and RLA rats demonstrated levels of ACh which were negatively correlated with their behavioural performance, thus the RLA animals with the highest level of ACh showed the poorest avoidance conditioning and lowest spontaneous activity levels, whilst the RHA showed lowest ACh but highest behavioural performance. The Porton strain was intermediate in both aspects. (AChE activities in whole brains were equal in the strains and it is therefore possible to refer to ACh concentrations as 'high' or 'low', where these levels are relative to AChE activity). Treatments which tended to increase ACh concentration in brain, such as treatment with anti-ChE drugs, consistently reduced performance of behaviour measured in all the strains. Conversely, alteration of cholinergic activity by antagonising its actions at central muscarinic sites, produced increased spontaneous activity and facilitated avoidance conditioning. In fact, the behavioural effects seen after increased ACh activity were exactly the opposite of those seen after ACh antagonism. These findings strongly suggest a role of ACh in the brain as an inhibitor of behavioural performance. (It is convenient to refer to behavioural performance in this context, where spontaneous activity level and avoidance conditioning are assumed to be positively related and constitute the measure of 'performances'). Enhanced behavioural performance of an apparently similar type, was produced by the non-cholinergic drug d-Amphetamine, suggesting that the cholinergic and adrenergic systems may play a joint role in control of behaviour. This suggestion received strong support when it was shown that small doses of anti-ACh and adrenergic drugs, given

together, were capable of producing improvements in performance that were greater than those seen when these doses of the drugs were given separately. Thus if the cholinergic system can be viewed as an inhibitor of behavioural performance, then the adrenergic system may be acting as its excitatory counterpart. Such a system for behavioural control has, of course, been proposed already be Carlton (1963, 1968, 1969), who has also proposed, as an extension to this theory, that cholinergic mechanisms may be involved, in particular, in the inhibition of non-reinforced behaviour. The present results appear to confirm and extend some of these ideas and permit some understanding of the nature of the differences that exist between the strains.

7.52 If the behaviour of the Roman strains is now re-examined it may be possible to describe their learning differences in terms of cholinergic inhibition. The RLA strain not only show low spontaneous activity but in particular, low exploratory activity, i.e., they rapidly accept new surroundings whilst RHA rats tend to spend much longer examining them and therefore show higher activity scores in the first part of the activity recordings. Novel stimuli, such as those present in a new environment attract the attention of animals for a period of time proportional to the significance they hold. Responses, such as sniffing, licking and touching rapidly disappear if the stimulus provides no reinforcement to cause them to continue. This fading of the response is known as habituation. It is suggested by Carlton (1968) that habituation is dependent upon the activity of brain ACh, therefore, RLA rats may be expected to habituate to the novelty of the activity cage very rapidly because they have high levels of ACh. Conversely, the RHA animals will continue to explore the activity cage for a longer period because their level of ACh inhibition is less.

An alternative explanation for the reduced activity of the RLA

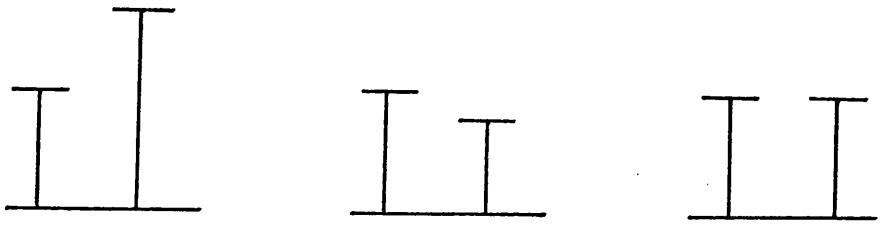
rats, is that it may be an emotional reaction to the new and therefore initially stressful situation. This may be discounted because Broadhurst and Bignami (1965) showed no differences between these strains when compared for emotional reactivity in an open-field test.

It may be possible to extend the idea of cholinergic inhibition of non-reinforced responses and imagine a continuum of ACh concentration with behavioural inhibition on which, (i) a very high level of ACh, such as would be present after treatment with anti-ChE drugs, and to slightly lesser extent, in the normal RLA rat, produces inhibition of all responses, reinforced or not, (ii) lower levels of ACh, (but still higher than normal) such as those present after a small dose of physostigmine, produce inhibition of non-reinforced responses leading to enhanced learning. This level is possibly very critical and the effect is probably only visible in certain types of tests, including, for example, maze-tests. Maze-learning involves many choices (responses) and slightly increased inhibition of non-reinforced choices, i.e., those which do not lead to the goal, may facilitate learning; (iii) slightly less than normal ACh activity, provides less inhibition leading to greater spontaneous activity and hyper-responsiveness to stimuli of all types, presumably associated with dominance of the adrenergic system. In some test situations where discrimination ability is the criterion for learning (for example, maze-tests), this state may produce poorer learning (Cox and Tye, 1973) but in others, such as the shuttlebox used in these experiments, enhancement is seen. The latter test contrasts with a maze test, in that good performance depends less upon making correct choices. The escape response is an obvious one, even to untrained rats and also the range of irrelevant, and therefore unreinforced, responses that may be indulged in by the rat during conditioning is limited. Finally, hyperactivity, the corollary of reduced cholinergic inhibition, tends to fac-

ilitate shuttlebox conditioning.

7.53 Strain differences in drug response may be a further manifestation of cholinergic inhibition. A closer examination of some of the strain differences in response to drugs may permit a finer analysis of the likely mechanisms involved. Strain differences in the response to d-Amphetamine suggest differences in catecholamine functions of the Roman rats, but Coyle et al (1973) found no differences in noradrenaline (NorA) turnover or the activities of enzymes of catecholamine synthesis. The differences in response to a-Amphetamine, therefore, may arise from a change in the relative activities of adrenergic and cholinergic systems. If the activity of the adrenergic component is similar in the Roman strain then the change in ACh concentration may be sufficient to explain the behavioural differences in response to d-Amphetamine.

Examination of another strain difference in response to drugs permits a further analysis of the controlling mechanisms. Physostigmine produced a depression of spontaneous activity of greater magnitudes in RHA strain rats than in RLA rats. The latter showed a smaller, but more prolonged depression. (See Figure 4-7). The cholinergic component of the interacting systems appears to be the dominant one in the RLA strain and increased ACh concentration after physostigmine may not produce extensive decrements in spontaneous activity because near maximal cholinergic effects may already be operative. The rapid and extensive depression seen in RHA rats, however, suggests that the balance between cholinergic inhibition and adrenergic excitation, here, is such that although the latter is dominant, a small rise in ACh has a dramatic effect in reversing the dominance. Thus the ratio of activities of one system to the other may be large in the RLA and small in the RHA strain, as shown below:



RLA RHA Porton
or Roman Control?

It is tentatively suggested that the Porton strain may show an approximately equal balance of the two systems although drug responses by the Porton strain are often of a different type to either Roman strain and there is little support for this suggestion. The arrangement shown on the right of the diagram, therefore, may be more typical of the strain of rat from which the Roman strains were bred (Roman control).

It is more difficult to analyse the Roman strains performance in the shuttlebox after physostigmine in this context because of the different types of performance that they show in this test. Generally, the performance of RHA rats was reduced to a greater extent compared to control than that of the RLA rats and thus may further support the suggested arrangement. On the other hand, the response decrements seen with the RHA strain were never greater than those seen with the Porton strain, whereas the suggestion ratio of adrenergic-cholinergic activities would anticipate the poorest performance from the latter. It is difficult to explain this discrepancy, except in terms of a typical responding by the Porton strain, reference to which has already been made.

7.54 Reducing the effects of cholinergic inhibition with NEPB or stimulating the adrenergic mechanisms with d-Amphetamine produced greater effects in the RLA strain than in either of the other strain. It might have been anticipated that larger doses of these drugs would be required

to produce hyperactivity or facilitated shuttlebox avoidance in the RLA strain than in the other strains, but this was not the case. Possibly, the RHA and Porton rats are close to the maximal performance in these parameters and show smaller percentage improvement for this reason. Similarly, when the drug combination of NEPB and d-Amphetamine was given, facilitation of behavioural performance was greatest in the RLA strain and second in the Porton strain, the RHA rats already show very high avoidance responding. Although the RLA rats were improved with all treatments that tended to reduce inhibition or stimulate the adrenergic system they never reached high levels of responding. Higher doses or different ratios of doses of anti-ACh and adrenergic may produce greater improvement but it may be necessary to investigate the contribution of other factors in producing their low responding.

7.55 Reference has already been made to the role of cholinergic inhibition and the part that habituation might play in exploratory activity. Another aspect of non-reinforced responding investigated in this work, was extinction of avoidance responding. Extinction may be compared to habituation as a behavioural measure. Learning to ignore stimuli which are of no significance, such as novel, but relevant objects in the environment (habituation) or an erstwhile conditioned stimulus in a training situation (extinction), is common to both forms of behaviour. If cholinergic inhibition has been shown to be involved in habituation, then it may also be expected to play a part in extinction behaviour. Carlton (1963, 1968, 1969) has shown this to be true. Thus treatment of rats trained in a conditioned avoidance response with an anti-ACh drug, delays the extinction period of that response, i.e., although the conditioned stimulus is no longer followed by shock (unreinforced), subjects with reduced brain ACh activity, continue to respond. In this work, RHA rats were found to have much longer times to extinction than Porton rats.

The Porton rats possess a greater concentration of ACh in whole brain than that of the RHA rats but the difference is not significant. Nevertheless, it is possible that there are different activities of ACh in smaller discrete areas of the brain undetected in these studies, which may be responsible for reduced cholinergic inhibition of the non-reinforced shuttlebox responding in the RHA rats. Differences between the strain were large, however, when the anti-ACh drug was given in extinction experiments. Both strains showed much delayed extinction, confirming Carlton's findings (1963, 1968, 1969), but the RHA strain continued to make conditioned responses for the whole of the duration of the experiment whereas the Porton stopped responding much sooner. This finding suggests that differences in cholinergic activity of the strains exists despite the lack of significant differences in ACh concentration, found in this work. The continued responding of the RHA strain probably resulted from the effects of adrenergic dominance after the cholinergic system was antagonised, producing enhanced responding of all types of behaviour. Thus avoidance responding to the conditioned stimulus would remain unchanged and responses to all other stimuli, including the shuttlebox itself, would be enhanced and maintained. It has been observed (this author, unpublished work) that rats treated with relatively high doses of centrally acting anti-ACh drugs frequently show not only hyperactivity but also an increased responsiveness to many types of stimuli. The cage activity consists of intensive exploratory activity and rats repeatedly jump or flinch in response to sudden noises or movements near the cage. This behaviour in RHA rats may possibly be seen with lower doses than are required to produce the effect in other rats, because of endogenous low ACh leading to reduced cholinergic inhibition.

7.56 The results of this work appear to support and extend the ideas which propose that certain aspects of behavioural control result from

the interaction of two biochemical systems, in the C.N.S., one of which is cholinergic and the other probably, is adrenergic. In this system the cholinergic components acts as an inhibitor of certain aspects of behaviour, especially, it would appear, on non-reinforced behaviour. Clearly, the system, as it has been described, is an over simplification and no attempts have been made to describe how this mechanism might be effected at the removal level nor what other systems might be involved. It is reasonable to suppose that there are other systems with which the proposed system interacts. A dopaminergic-cholinergic interaction has already been described in the C.N.S. (Hornykiewicz, 1968), the inbalance of which may be associated with parkinsonism.

7.57 The use of selectively bred strains proved a useful approach in this research. The strains by showing distinct and consistent differences in behaviour provided stable measures against which drug effects could be assessed. Differences in existing biochemical systems which correlated with behaviour permitted an analysis of drug action which would not have been possible with non-selected strains of rat. It is considered, therefore, that selectively bred strains may be a useful tool in many aspects of psychopharmacological research. In routine screening of drugs for psychopharmacological effects, selectively bred strains could serve the useful function of providing consistent behavioural responses. This aspect of their use is particularly attractive because many routine behavioural techniques are hampered by the wide range of variation in the behavioural performance which is present in most population of laboratory animals. Care should be exercised, however, in the use of experimental subjects, selectively bred to perform consistently well or consistently badly, in a particular test situation. The most useful selectively bred animals for pharmacological research are those which, not only exhibit the desired behavioural response, but also do so because of a similar

mechanism to that which controls the behaviour in the original animal stock. Thus when selective breeding is performed, great care must be taken to ensure that none but the desired characters are selected. The specificity of selective breeding has been described- thus the Tryon strains only exhibited maze-learning differences when trained in the maze and by the same detailed procedure, as was used during their selection (Searle 1949). Similarly, the Roman strains show large differences in shuttlebox avoidance behaviour but much smaller differences in learning ability when compared in a closely related avoidance test, a pole-jump test (Davies, personal communication, 1973). Selective breeding therefore must be conducted with great care, but when this is done it may be possible to obtain animals for research which embody in exaggerated form the outward characters of interest, and hopefully also, the biochemical mechanisms which controlled it in the original population.

7.6 *Suggestions for Further Work*

Many new lines of research may be suggested by the work done here but only a few of the most potentially interesting aspects of these will be included here.

Although the choice of the Roman strains for this work proved useful, it had been hoped that the Porton strain would also prove useful as a reference strain with which the Roman strains might be compared; unfortunately, the usefulness of the Porton strain in this respect was limited. Differences in response to drugs suggested that this strain differed from the Roman strain in certain basic characters which were not necessarily the result of the selective breeding experiments. More useful, therefore, in further work of this kind, would be a control strain which arose from the parent stock of the selected strains.

The performance of RLA rats in shuttlebox conditioning in these experiments, was such that only during certain drug studies was avoidance

responding seen. It is possible that a modified shuttlebox or a different conditioning test which involved shock and an active response, may permit some learning by this strain. Comparisons of strain behaviour would thus be facilitated and the information gained may permit a closer analysis of their differences.

A facility for counting intertrial responses during shuttlebox training may be considered a useful addition to the type of equipment used in this work. It was seen that in experiments involving hyperactivity of the subject, a more accurate assessment of conditioning would be possible if the rate of intertrial responding was known.

Further biochemical studies of brain transmitter systems are obviated to clarify the roles of cholinergic, adrenergic and other substances in the control of the behaviour of these strains and thus provide information concerning behaviour control in general.

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